ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

World Journal of *Gastroenterology*

World J Gastroenterol 2014 January 28; 20(4): 869-1126

Baishideng Publishing Group
www.wjgnet.com

World Journal of Gastroenterology

A peer-reviewed, online, open-access journal of gastroenterology and hepatology

Editorial Board

2014-2017

The *World Journal of Gastroenterology* Editorial Board consists of 1339 members, representing a team of worldwide experts in gastroenterology and hepatology. They are from 67 countries, including Albania (1), Argentina (7), Australia (31), Austria (9), Belgium (10), Brazil (20), Brunei Darussalam (1), Bulgaria (2), Cambodia (1), Canada (25), Chile (4), China (158), Croatia (1), Cuba (1), Czech (6), Denmark (2), Egypt (9), Estonia (2), Finland (5), France (17), Germany (56), Greece (31), Guatemala (1), Hungary (14), Iceland (1), India (32), Indonesia (2), Iran (9), Ireland (9), Israel (17), Italy (193), Japan (151), Jordan (1), Kuwait (1), Lebanon (7), Lithuania (1), Malaysia (1), Mexico (10), Morocco (1), Netherlands (5), New Zealand (4), Nigeria (3), Norway (6), Pakistan (6), Poland (12), Portugal (8), Puerto Rico (1), Qatar (1), Romania (9), Russia (3), Saudi Arabia (2), Singapore (7), Slovenia (2), South Korea (63), Spain (51), Sri Lanka (1), Sudan (1), Sweden (12), Switzerland (5), Thailand (7), Trinidad and Tobago (1), Tunisia (2), Turkey (56), United Kingdom (47), United States (171), Venezuela (1), and Vietnam (1).

Alfred Gangl, *Vienna*
Kurt Lenz, *Linz*
Markus Peck-Radosavljevic, *Vienna*
Markus Raderer, *Vienna*
Stefan Riss, *Vienna*

Belgium

Michael George Adler, *Brussels*
Benedicte Y De Winter, *Antwerp*
Mark De Ridder, *Jette*
Olivier Detry, *Liege*
Denis Dufrane Dufrane, *Brussels*
Nikos Kotzampassakis, *Liège*
Geert KMM Robaeys, *Genk*
Xavier Sagaert, *Leuven*
Peter Starkel, *Brussels*
Eddie Wisse, *Keerbergen*

Brazil

SMP Balzan, *Santa Cruz do Sul*
JLF Caboclo, *Sao jose do rio preto*
Fábio Guilherme Campos, *Sao Paulo*
Claudia RL Cardoso, *Rio de Janeiro*
Roberto J Carvalho-Filho, *Sao Paulo*
Carla Daltro, *Salvador*
José Sebastiao dos Santos, *Ribeirao Preto*
Eduardo LR Mello, *Rio de Janeiro*
Sthela Maria Murad-Regadas, *Fortaleza*
Claudia PMS Oliveira, *Sao Paulo*
Júlio C Pereira-Lima, *Porto Alegre*
Marcos V Perini, *Sao Paulo*
Vietla Satyanarayana Rao, *Fortaleza*
Raquel Rocha, *Salvador*
AC Simoes e Silva, *Belo Horizonte*
Mauricio F Silva, *Porto Alefre*
Aytan Miranda Sipahi, *Sao Paulo*
Rosa Leonôra Salerno Soares, *Niterói*
Cristiane Valle Tovo, *Porto Alegre*
Eduardo Garcia Vilela, *Belo Horizonte*

Brunei Darussalam

Vui Heng Chong, *Bandar Seri Begawan*

Bulgaria

Tanya Kirilova Kadiyska, *Sofia*
Mihaela Petrova, *Sofia*

Cambodia

Francois Rouet, *Phnom Penh*

Canada

Brian Bressler, *Vancouver*
Frank J Burczynski, *Winnipeg*
Wangxue Chen, *Ottawa*
Francesco Crea, *Vancouver*
Mirko Diksic, *Montreal*
Jane A Foster, *Hamilton*
Hugh J Freeman, *Vancouver*
Shahrokh M Ghobadloo, *Ottawa*
Yuewen Gong, *Winnipeg*
Philip H Gordon, *Quebec*
Rakesh Kumar, *Edmonton*
Wolfgang A Kunze, *Hamilton*
Patrick Labonte, *Laval*
Zhikang Peng, *Winnipeg*
Jayadev Raju, *Ottawa*
Maitreyi Raman, *Calgary*
Giada Sebastiani, *Montreal*
Maida J Sewitch, *Montreal*
Eldon A Shaffer, *Alberta*
Christopher W Teshima, *Edmonton*
Jean Sévigny, *Québec*
Pingchang Yang, *Hamilton*
Pingchang Yang, *Hamilton*
Eric M Yoshida, *Vancouver*
Bin Zheng, *Edmonton*

Chile

Marcelo A Beltran, *La Serena*
Flavio Nervi, *Santiago*
Adolfo Parra-Blanco, *Santiago*
Alejandro Soza, *Santiago*

China

Zhao-Xiang Bian, *Hong Kong*
San-Jun Cai, *Shanghai*
Guang-Wen Cao, *Shanghai*
Long Chen, *Nanjing*
Ru-Fu Chen, *Guangzhou*
George G Chen, *Hong Kong*
Li-Bo Chen, *Wuhan*
Jia-Xu Chen, *Beijing*
Hong-Song Chen, *Beijing*
Lin Chen, *Beijing*
Yang-Chao Chen, *Hong Kong*
Zhen Chen, *Shanghai*
Ying-Sheng Cheng, *Shanghai*
Kent-Man Chu, *Hong Kong*
Zhi-Jun Dai, *Xi'an*
Jing-Yu Deng, *Tianjin*
Yi-Qi Du, *Shanghai*
Zhi Du, *Tianjin*
Hani El-Nezami, *Hong Kong*
Bao-Ying Fei, *Hangzhou*
Chang-Ming Gao, *Nanjing*
Jian-Ping Gong, *Chongqing*
Zuo-Jiong Gong, *Wuhan*
Jing-Shan Gong, *Shenzhen*
Yong-Song Guan, *Chengdu*
Mao-Lin Guo, *Luoyang*
Jun-Ming Guo, *Ningbo*
Yan-Mei Guo, *Shanghai*
Xiao-Zhong Guo, *Shenyang*
Guo-Hong Han, *Xi'an*
Ming-Liang He, *Hong Kong*
Peng Hou, *Xi'an*
Zhao-Hui Huang, *Wuxi*
Feng Ji, *Hangzhou*
Simon Law, *Hong Kong*
Yu-Yuan Li, *Guangzhou*
Meng-Sen Li, *Haikou*
Shu-De Li, *Shanghai*
Zong-Fang Li, *Xi'an*
Qing-Quan Li, *Shanghai*
Kang Li, *Lasa*
Han Liang, *Tianjin*
Xing'e Liu, *Hangzhou*
Zheng-Wen Liu, *Xi'an*
Xiao-Fang Liu, *Yantai*
Bin Liu, *Tianjin*
Quan-Da Liu, *Beijing*
Hai-Feng Liu, *Beijing*
Fei Liu, *Shanghai*
Ai-Guo Lu, *Shanghai*
He-Sheng Luo, *Wuhan*
Xiao-Peng Ma, *Shanghai*
Yong Meng, *Shantou*
Ke-Jun Nan, *Xi'an*
Siew Chien Ng, *Hong Kong*
Simon SM Ng, *Hong Kong*
Zhao-Shan Niu, *Qingdao*
Bo-Rong Pan, *Xi'an*
Di Qu, *Shanghai*
Rui-Hua Shi, *Nanjing*
Bao-Min Shi, *Shanghai*
Xiao-Dong Sun, *Hangzhou*
Guang-Hong Tan, *Haikou*
Wen-Fu Tang, *Chengdu*
Anthony YB Teoh, *Hong Kong*
Wei-Dong Tong, *Chongqing*
Eric Tse, *Hong Kong*
Hong Tu, *Shanghai*
Rong Tu, *Haikou*
Jian-She Wang, *Shanghai*
Kai Wang, *Jinan*
Xiao-Ping Wang, *Xianyang*
Dao-Rong Wang, *Yangzhou*
De-Sheng Wang, *Xi'an*
Chun-You Wang, *Wuhan*
Ge Wang, *Chongqing*
Xi-Shan Wang, *Harbin*
Wei-hong Wang, *Beijing*
Wai Man Raymond Wong, *Hong Kong*
Chun-Ming Wong, *Hong Kong*
Jian Wu, *Shanghai*
Sheng-Li Wu, *Xi'an*
Wu-Jun Wu, *Xi'an*
Bing Xia, *Wuhan*
Qing Xia, *Chengdu*
Yan Xin, *Shenyang*
Dong-Ping Xu, *Beijing*
Jian-Min Xu, *Shanghai*
Wei Xu, *Changchun*
Ming Yan, *Jinan*
Xin-Min Yan, *Kunming*
Yi-Qun Yan, *Shanghai*
Feng Yang, *Shanghai*
Yong-Ping Yang, *Beijing*
He-Rui Yao, *Guangzhou*
Thomas Yau, *Hong Kong*
Winnie Yeo, *Hong Kong*
Jing You, *Kunming*
Jian-Qing Yu, *Wuhan*
Ying-Yan Yu, *Shanghai*
Wei-Zheng Zeng, *Chengdu*
Zong-Ming Zhang, *Beijing*
Dian-Liang Zhang, *Qingdao*
Ya-Ping Zhang, *Shijiazhuang*
You-Cheng Zhang, *Lanzhou*
Jian-Zhong Zhang, *Beijing*
Ji-Yuan Zhang, *Beijing*
Hai-Tao Zhao, *Beijing*
Jian Zhao, *Shanghai*
Jian-Hong Zhong, *Nanning*

Ying-Qiang Zhong, *Guangzhou*
Ping-Hong Zhou, *Shanghai*
Yan-Ming Zhou, *Xiamen*
Tong Zhou, *Nanchong*
Li-Ming Zhou, *Chengdu*
Guo-Xiong Zhou, *Nantong*
Feng-Shang Zhu, *Shanghai*
Jiang-Fan Zhu, *Shanghai*
Zhao-Hui Zhu, *Beijing*

Croatia

Tajana Filipec Kanizaj, *Zagreb*

Cuba

Damian Casadesus, *Havana*

Czech

Jan Bures, *Hradec Kralove*
Marcela Kopacova, *Hradec Kralove*
Otto Kucera, *Hradec Kralove*
Marek Minarik, *Prague*
Pavel Soucek, *Prague*
Miroslav Zavoral, *Prague*

Denmark

Vibeke Andersen, *Odense*
E Michael Danielsen, *Copenhagen*

Egypt

Mohamed MM Abdel-Latif, *Assiut*
Hussein Atta, *Cairo*
Ashraf Elbahrawy, *Cairo*
Mortada Hassan El-Shabrawi, *Cairo*
Mona El Said El-Raziky, *Cairo*
Elrashdy M Redwan, *New Borg Alrab*
Zeinab Nabil Ahmed Said, *Cairo*
Ragaa HM Salama, *Assiut*
Maha Maher Shehata, *Mansoura*

Estonia

Margus Lember, *Tartu*
Tamara Vorobjova, *Tartu*

Finland

Marko Kalliomäki, *Turku*
Thomas Kietzmann, *Oulu*
Kaija-Leena Kolho, *Helsinki*
Eija Korkeila, *Turku*
Heikki Makisalo, *Helsinki*

France

Armando Abergel Clermont, *Ferrand*
Elie K Chouillard, *Polssy*
Pierre Cordelier, *Toulouse*
Pascal P Crenn, *Garches*
Catherine Daniel, *Lille*
Fanny Daniel, *Paris*
Cedric Dray, *Toulouse*
Benoit Foligne, *Lille*
Jean-Noel Freund, *Strasbourg*
Nathalie Janel, *Paris*
Majid Khatib, *Bordeaux*
Jacques Marescaux, *Strasbourg*
Jean-Claude Marie, *Paris*
Hang Nguyen, *Clermont-Ferrand*
Hugo Perazzo, *Paris*
Alain L Servin, *Chatenay-Malabry*
Chang Xian Zhang, *Lyon*

Germany

Stavros A Antoniou, *Monchengladbach*
Erwin Biecker, *Siegburg*
Hubert E Blum, *Freiburg*
Thomas Bock, *Berlin*
Katja Breitkopf-Heinlein, *Mannheim*
Elke Cario, *Essen*
Güralp Onur Ceyhan, *Munich*
Angel Cid-Arregui, *Heidelberg*
Michael Clemens Roggendorf, *München*
Christoph F Dietrich, *Bad Mergentheim*
Valentin Fuhrmann, *Hamburg*
Nikolaus Gassler, *Aachen*
Andreas Geier, *Wuerzburg*
Markus Gerhard, *Munich*
Anton Gillessen, *Muenster*
Thorsten Oliver Goetze, *Offenbach*
Daniel Nils Gotthardt, *Heidelberg*
Robert Grützmann, *Dresden*
Thilo Hackert, *Heidelberg*
Joerg Haier, *Muenster*
Claus Hellerbrand, *Regensburg*
Harald Peter Hoensch, *Darmstadt*
Jens Hoeppner, *Freiburg*
Richard Hummel, *Muenster*
Jakob Robert Izbicki, *Hamburg*
Gernot Maximilian Kaiser, *Essen*
Matthias Kapischke, *Hamburg*
Michael Keese, *Frankfurt*
Andrej Khandoga, *Munich*
Jorg Kleeff, *Munich*
Alfred Koenigsrainer, *Tuebingen*
Peter Christopher Konturek, *Saalfeld*
Michael Linnebacher, *Rostock*
Stefan Maier, *Kaufbeuren*
Oliver Mann, *Hamburg*
Marc E Martignoni, *Munic*
Thomas Minor, *Bonn*
Oliver Moeschler, *Osnabrueck*
Jonas Mudter, *Eutin*
Sebastian Mueller, *Heidelberg*
Matthias Ocker, *Berlin*
Andreas Ommer, *Essen*
Albrecht Piiper, *Frankfurt*
Esther Raskopf, *Bonn*
Christoph Reichel, *Bad Brückenau*
Elke Roeb, *Giessen*
Udo Rolle, *Frankfurt*
Karl-Herbert Schafer, *Zweibrücken*
Andreas G Schreyer, *Regensburg*
Manuel A Silva, *Penzberg*
Georgios C Sotiropoulos, *Essen*
Ulrike S Stein, *Berlin*
Dirk Uhlmann, *Leipzig*
Michael Weiss, *Halle*
Hong-Lei Weng, *Mannheim*
Karsten Wursthorn, *Hamburg*

Greece

Alexandra Alexopoulou, *Athens*
Nikolaos Antonakopoulos, *Athens*
Stelios F Assimakopoulos, *Patras*
Grigoris Chatzimavroudis, *Thessaloniki*
Evangelos Cholongitas, *Thessaloniki*
Gregory Christodoulidis, *Larisa*
George N Dalekos, *Larissa*
Maria Gazouli, *Athens*
Urania Georgopoulou, *Athens*
Eleni Gigi, *Thessaloniki*
Stavros Gourgiotis, *Athens*
Leontios J Hadjileontiadis, *Thessaloniki*
Thomas Hyphantis, *Ioannina*
Ioannis Kanellos, *Thessaloniki*
Stylianos Karatapanis, *Rhodes*
Michael Koutsilieris, *Athens*
Spiros D Ladas, *Athens*
Theodoros K Liakakos, *Athens*
Emanuel K Manesis, *Athens*
Spilios Manolakopoulos, *Athens*
Gerassimos John Mantzaris, *Athens*
Athanasios D Marinis, *Piraeus*
Nikolaos Ioannis Nikiteas, *Athens*
Konstantinos X Papamichael, *Athens*
George Sgourakis, *Athens*
Konstantinos C Thomopoulos, *Patras*
Konstantinos Triantafyllou, *Athens*
Christos Triantos, *Patras*
Georgios Zacharakis, *Athens*
Petros Zezos, *Alexandroupolis*
Demosthenes E Ziogas, *Ioannina*

Guatemala

Carlos Maria Parellada, *Guatemala*

Hungary

Mihaly Boros, *Szeged*
Tamás Decsi, *Pécs*
Gyula Farkas, *Szeged*
Andrea Furka, *Debrecen*
Y vette Mandi, *Szeged*
Peter L Lakatos, *Budapest*
Pal Miheller, *Budapest*
Tamás Molnar, *Szeged*
Attila Olah, *Gyor*
Maria Papp, *Debrecen*
Zoltan Rakonczay, *Szeged*
Ferenc Sipos, *Budapest*
Miklós Tanyi, *Debrecen*
Tibor Wittmann, *Szeged*

Iceland

Tryggvi Bjorn Stefánsson, *Reykjavík*

India

Brij B Agarwal, *New Delhi*
Deepak N Amarapurkar, *Mumbai*
Shams ul Bari, *Srinagar*
Sriparna Basu, *Varanasi*
Devendra C Desai, *Mumbai*
Nutan D Desai, *Mumbai*
Suneela Sunil Dhaneshwar, *Pune*
Radha K Dhiman, *Chandigarh*
Pankaj Garg, *Mohali*
Uday C Ghoshal, *Lucknow*
Kalpesh Jani, *Vadodara*
Premashis Kar, *New Delhi*
Jyotdeep Kaur, *Chandigarh*
Rakesh Kochhar, *Chandigarh*
Pradyumna K Mishra, *Mumbai*
Asish K Mukhopadhyay, *Kolkata*
Imtiyaz Murtaza, *Srinagar*
P Nagarajan, *New Delhi*
Samiran Nundy, *Delhi*
Gopal Pande, *Hyderabad*
Benjamin Perakath, *Vellore*
Arun Prasad, *New Delhi*
D Nageshwar Reddy, *Hyderabad*
Lekha Saha, *Chandigarh*
Sundeep Singh Saluja, *New Delhi*
Mahesh Prakash Sharma, *New Delhi*
Sadiq Saleem Sikora, *Bangalore*
Sarman Singh, *New Delhi*
Rajeev Sinha, *Jhansi*
Rupjyoti Talukdar, *Hyderabad*
Rakesh Kumar Tandon, *New Delhi*
Narayanan Thirumoorthy, *Coimbatore*

Indonesia

David Handojo Muljono, *Jakarta*
Andi Utama, *Jakarta*

Iran

Arezoo Aghakhani, *Tehran*
Seyed Mohsen Dehghani, *Shiraz*
Hossein Khedmat, *Tehran*
Sadegh Massarrat, *Tehran*
Marjan Mohammadi, *Tehran*
Roja Rahimi, *Tehran*
Farzaneh Sabahi, *Tehran*
Majid Sadeghizadeh, *Tehran*
Farideh Siavoshi, *Tehran*

Ireland

Gary Alan Bass, *Dublin*
David J Brayden, *Dublin*
Ronan A Cahill, *Dublin*
Glen A Doherty, *Dublin*
Liam J Fanning, *Cork*
Barry Philip McMahon, *Dublin*
RossMcManus, *Dublin*
Dervla O'Malley, *Cork*
Sinead M Smith, *Dublin*

Israel

Dan Carter, *Ramat Gan*
Eli Magen, *Ashdod*
Nitsan Maharshak, *Tel Aviv*
Shaul Mordechai, *Beer Sheva*
Menachem Moshkowitz, *Tel Aviv*
William Bahij Nseir, *Nazareth*
Shimon Reif, *Jerusalem*
Ram Reifen, *Rehovot*
Ariella Bar-Gil Shitrit, *Jerusalem*
Noam Shussman, *Jerusalem*
Igor Sukhotnik, *Haifa*
Nir Wasserberg, *Petach Tiqwa*
Jacob Yahav, *Rehovot*
Doron Levi Zamir, *Gedera*
Shira Zelber-Sagi, *Haifa*
Romy Zemel, *Petach-Tikva*

Italy

Ludovico Abenavoli, *Catanzaro*
Luigi Elio Adinolfi, *Naples*
Carlo Virginio Agostoni, *Milan*
Anna Alisi, *Rome*
Piero Luigi Almasio, *Palermo*
Donato Francesco Altomare, *Bari*
Amedeo Amedei, *Florence*
Pietro Andreone, *Bologna*
Imerio Angriman, *Padova*
Vito Annese, *Florence*
Paolo Aurello, *Rome*
Salavtore Auricchio, *Naples*
Gian Luca Baiocchi, *Brescia*
Gianpaolo Balzano, *Milan*
Antonio Basoli, *Rome*
Gabrio Bassotti, *San Sisto*
Mauro Bernardi, *Bologna*
Alberto Biondi, *Rome*
Ennio Biscaldi, *Genova*
Massimo Bolognesi, *Padua*
Luigi Bonavina, *Milano*
Aldo Bove, *Chieti*
Raffaele Bruno, *Pavia*
Luigi Brusciano, *Napoli*
Giuseppe Cabibbo, *Palermo*
Carlo Calabrese, *Bologna*
Daniele Calistri, *Meldola*
Vincenza Calvaruso, *Palermo*
Lorenzo Camellini, *Reggio Emilia*
Marco Candela, *Bologna*
Raffaele Capasso, *Naples*
Lucia Carulli, *Modena*
Renato David Caviglia, *Rome*
Luigina Cellini, *Chieti*
Giuseppe Chiarioni, *Verona*
Claudio Chiesa, *Rome*
Michele Cicala, *Roma*
Rachele Ciccocioppo, *Pavia*
Sandro Contini, *Parma*
Gaetano Corso, *Foggia*
Renato Costi, *Parma*
Alessandro Cucchetti, *Bologna*
Rosario Cuomo, *Napoli*
Giuseppe Currò, *Messina*
Paola De Nardi, *Milano*
Giovanni D De Palma, *Naples*
Raffaele De Palma, *Napoli*
Giuseppina De Petro, *Brescia*
Valli De Re, *Aviano*
Paolo De Simone, *Pisa*
Giuliana Decorti, *Trieste*
Emanuele Miraglia del Giudice, *Napoli*
Isidoro Di Carlo, *Catania*
Matteo Nicola Dario Di Minno, *Naples*
Massimo Donadelli, *Verona*
Mirko D'Onofrio, *Verona*
Maria Pina Dore, *Sassari*
Luca Elli, *Milano*
Massimiliano Fabozzi, *Aosta*
Massimo Falconi, *Ancona*
Ezio Falletto, *Turin*
Silvia Fargion, *Milan*
Matteo Fassan, *Verona*
Gianfranco Delle Fave, *Roma*
Alessandro Federico, *Naples*
Francesco Feo, *Sassari*
Davide Festi, *Bologna*
Natale Figura, *Siena*
Vincenzo Formica, *Rome*
Mirella Fraquelli, *Milan*
Marzio Frazzoni, *Modena*
Walter Fries, *Messina*
Gennaro Galizia, *Naples*
Andrea Galli, *Florence*
Matteo Garcovich, *Rome*
Eugenio Gaudio, *Rome*
Paola Ghiorzo, *Genoa*
Edoardo G Giannini, *Genova*
Luca Gianotti, *Monza*
Maria Cecilia Giron, *Padova*
Alberto Grassi, *Rimini*
Gabriele Grassi, *Trieste*
Francesco Greco, *Bergamo*
Luigi Greco, *Naples*
Antonio Grieco, *Rome*
Fabio Grizzi, *Rozzano*
Laurino Grossi, *Pescara*
Salvatore Gruttadauria, *Palermo*
Simone Guglielmetti, *Milan*
Tiberiu Hershcovici, *Jerusalem*
Calogero Iacono, *Verona*
Enzo Ierardi, *Bari*
Amedeo Indriolo, *Bergamo*
Raffaele Iorio, *Naples*
Paola Iovino, *Salerno*
Angelo A Izzo, *Naples*
Loreta Kondili, *Rome*
Filippo La Torre, *Rome*
Giuseppe La Torre, *Rome*
Giovanni Latella, *L'Aquila*
Salvatore Leonardi, *Catania*
Massimo Libra, *Catania*
Anna Licata, *Palermo*
C armela Loguercio, *Naples*
Amedeo Lonardo, *Modena*
Carmelo Luigiano, *Catania*
Francesco Luzza, *Catanzaro*
Giovanni Maconi, *Milano*
Antonio Macrì, *Messina*
Mariano Malaguarnera, *Catania*
Francesco Manguso, *Napoli*
Tommaso Maria Manzia, *Rome*
Daniele Marrelli, *Siena*
Gabriele Masselli, *Rome*

Sara Massironi, *Milan*
Giuseppe Mazzarella, *Avellino*
Michele Milella, *Rome*
Giovanni Milito, *Rome*
Antonella d'Arminio Monforte, *Milan*
Fabrizio Montecucco, *Genoa*
Giovanni Monteleone, *Rome*
Mario Morino, *Torino*
Vincenzo La Mura, *Milan*
Gerardo Nardone, *Naples*
Riccardo Nascimbeni, *Brescia*
Gabriella Nesi, *Florence*
Giuseppe Nigri, *Rome*
Erica Novo, *Turin*
Veronica Ojetti, *Rome*
Michele Orditura, *Naples*
Fabio Pace, *Seriate*
Lucia Pacifico, *Rome*
Omero Alessandro Paoluzi, *Rome*
Valerio Pazienza, *San Giovanni Rotondo*
Rinaldo Pellicano, *Turin*
Adriano M Pellicelli, *Rome*
Nadia Peparini, *Ciampino*
Mario Pescatori, *Rome*
Antonio Picardi, *Rome*
Alberto Pilotto, *Padova*
Alberto Piperno, *Monza*
Anna Chiara Piscaglia, *Rome*
Maurizio Pompili, *Rome*
Francesca Romana Ponziani, *Rome*
Cosimo Prantera, *Rome*
Girolamo Ranieri, *Bari*
Carlo Ratto, *Tome*
Barbara Renga, *Perugia*
Alessandro Repici, *Rozzano*
Maria Elena Riccioni, *Rome*
Lucia Ricci-Vitiani, *Rome*
Luciana Rigoli, *Messina*
Mario Rizzetto, *Torino*
Ballarin Roberto, *Modena*
Roberto G Romanelli, *Florence*
Claudio Romano, *Messina*
Luca Roncucci, *Modena*
Cesare Ruffolo, *Treviso*
L ucia Sacchetti, *Napoli*
Rodolfo Sacco, *Pisa*
Romina Salpini, *Rome*
Giulio Aniello, *Santoro Treviso*
Armando Santoro, *Rozzano*
Edoardo Savarino, *Padua*
Marco Senzolo, *Padua*
Annalucia Serafino, *Rome*
Giuseppe S Sica, *Rome*
Pierpaolo Sileri, *Rome*
Cosimo Sperti, *Padua*
Vincenzo Stanghellini, *Bologna*
Cristina Stasi, *Florence*
Gabriele Stocco, *Trieste*
Roberto Tarquini, *Florence*
Mario Testini, *Bari*
Guido Torzilli, *Milan*
Guido Alberto Massimo, *Tiberio Brescia*
Alberto Tommasini, *Trieste*
Francesco Tonelli, *Florence*
Cesare Tosetti Porretta, *Terme*
Lucio Trevisani, *Cona*
Guglielmo M Trovato, *Catania*
Mariapia Vairetti, *Pavia*
Luca Vittorio Valenti, *Milano*
Mariateresa T Ventura, *Bari*
Giuseppe Verlato, *Verona*
Alessandro Vitale, *Padova*
Marco Vivarelli, *Ancona*
Giovanni Li Volti, *Catania*
Giuseppe Zanotti, *Padua*
Vincenzo Zara, *Lecce*
Gianguglielmo Zehender, *Milan*
Anna Linda Zignego, *Florence*
Rocco Antonio Zoccali, *Messina*
Angelo Zullo, *Rome*

Japan

Yasushi Adachi, *Sapporo*
Takafumi Ando, *Nagoya*
Masahiro Arai, *Tokyo*
Makoto Arai, *Chiba*
Takaaki Arigami, *Kagoshima*
Itaru Endo,*Yokohama*
Munechika Enjoji, *Fukuoka*
Shunji Fujimori, *Tokyo*
Yasuhiro Fujino, *Akashi*
Toshiyoshi Fujiwara, *Okayama*
Yosuke Fukunaga, *Tokyo*
Toshio Fukusato, *Tokyo*
Takahisa Furuta, *Hamamatsu*
Osamu Handa, *Kyoto*
Naoki Hashimoto, *Osaka*
Yoichi Hiasa, *Toon*
Masatsugu Hiraki, *Saga*
Satoshi Hirano, *Sapporo*
Keiji Hirata, *Fukuoka*
Toru Hiyama, *Higashihiroshima*
Akira Hokama, *Nishihara*
Shu Hoteya, *Tokyo*
Masao Ichinose, *Wakayama*
Tatsuya Ide, *Kurume*
Masahiro Iizuka, *Akita*
Toshiro Iizuka, *Tokyo*
Kenichi Ikejima, *Tokyo*
Tetsuya Ikemoto, *Tokushima*
Hiroyuki Imaeda, *Saitama*
Atsushi Imagawa, *Kan-onji*
Hiroo Imazu, *Tokyo*
Akio Inui, *Kagoshima*
Shuji Isaji, *Tsu*
Toru Ishikawa, *Niigata*
Toshiyuki Ishiwata, *Tokyo*
Soichi Itaba, *Kitakyushu*
Yoshiaki Iwasaki, *Okayama*
Tatehiro Kagawa, *Isehara*
Satoru Kakizaki, *Maebashi*
Naomi Kakushima, *Shizuoka*
Terumi Kamisawa, *Tokyo*
Akihide Kamiya, *Isehara*
Osamu Kanauchi, *Tokyo*
Tatsuo Kanda, *Chiba*
Shin Kariya, *Okayama*
Shigeyuki Kawa, *Matsumoto*
Takumi Kawaguchi, *Kurume*
Takashi Kawai, *Tokyo*
Soo Ryang Kim, *Kobe*
Shinsuke Kiriyama, *Gunma*
Tsuneo Kitamura, *Urayasu*
Masayuki Kitano, *Osakasayama*
Hirotoshi Kobayashi, *Tokyo*
Hironori Koga, *Kurume*
Takashi Kojima, *Sapporo*
Satoshi Kokura, *Kyoto*
Shuhei Komatsu, *Kyoto*
Tadashi Kondo, *Tokyo*
Yasuteru Kondo, *Sendai*
Yasuhiro Kuramitsu, *Yamaguchi*
Yukinori Kurokawa, *Osaka*
Shin Maeda, *Yokohama*
Koutarou Maeda, *Toyoake*
Hitoshi Maruyama, *Chiba*
Atsushi Masamune, *Sendai*
Hiroyuki Matsubayashi, *Suntogun*
Akihisa Matsuda, *Inzai*
Hirofumi Matsui, *Tsukuba*
Akira Matsumori, *Kyoto*
Yoichi Matsuo, *Nagoya*
Y Matsuzaki, *Ami*
Toshihiro Mitaka, *Sapporo*
Kouichi Miura, *Akita*
Shinichi Miyagawa, *Matumoto*
Eiji Miyoshi, *Suita*
Toru Mizuguchi, *Sapporo*
Nobumasa Mizuno, *Nagoya*
Zenichi Morise, *Nagoya*
Tomohiko Moriyama, *Fukuoka*
Kunihiko Murase, *Tusima*
Michihiro Mutoh, *Tsukiji*
Akihito Nagahara, *Tokyo*
Hikaru Nagahara, *Tokyo*
Hidenari Nagai, *Tokyo*
Koichi Nagata, *Shimotsuke-shi*
Masaki Nagaya, *Kawasaki*
Hisato Nakajima, *Nishi-Shinbashi*
Toshifusa Nakajima, *Tokyo*
Hiroshi Nakano, *Kawasaki*
Hiroshi Nakase, *Kyoto*
Toshiyuki Nakayama, *Nagasaki*
Takahiro Nakazawa, *Nagoya*
Shoji Natsugoe, *Kagoshima City*
Tsutomu Nishida, *Suita*
Shuji Nomoto, *Naogya*
Sachiyo Nomura, *Tokyo*
Takeshi Ogura, *Takatsukishi*
Nobuhiro Ohkohchi, *Tsukuba*
Toshifumi Ohkusa, *Kashiwa*
Hirohide Ohnishi, *Akita*
Teruo Okano, *Tokyo*
Satoshi Osawa, *Hamamatsu*
Motoyuki Otsuka, *Tokyo*
Michitaka Ozaki, *Sapporo*
Satoru Saito, *Yokohama*
Chouhei Sakakura, *Kyoto*
Naoaki Sakata, *Sendai*
Ken Sato, *Maebashi*
Toshiro Sato, *Tokyo*
Tomoyuki Shibata, *Toyoake*
H Shimada, *Tokyo*
Tomohiko Shimatani, *Kure*
Yukihiro Shimizu, *Nanto*
Tadashi Shimoyama, *Hirosaki*
Masayuki Sho, *Nara*
Ikuo Shoji, *Kobe*
Atsushi Sofuni, *Tokyo*
Takeshi Suda, *Niigata*
M Sugimoto, *Hamamatsu*
Ken Sugimoto, *Hamamatsu*
Haruhiko Sugimura, *Hamamatsu*
Shoichiro Sumi, *Kyoto*
Hidekazu Suzuki, *Tokyo*
Masahiro Tajika, *Nagoya*
Hitoshi Takagi, *Takasaki*
Toru Takahashi, *Niigata*
Yoshihisa Takahashi, *Tokyo*
Shinsuke Takeno, *Fukuoka*
Akihiro Tamori, *Osaka*
Kyosuke Tanaka, *Tsu*
Shinji Tanaka, *Hiroshima*

Atsushi Tanaka, *Tokyo*
Yasuhito Tanaka, *Nagoya*
Shinji Tanaka, *Tokyo*
Minoru Tomizawa, *Yotsukaido City*
Kyoko Tsukiyama-Kohara, *Kagoshima*
Takuya Watanabe, *Niigata*
Kazuhiro Watanabe, *Sendai*
Satoshi Yamagiwa, *Niigata*
Takayuki Yamamoto, *Yokkaichi*
Hiroshi Yamamoto, *Otsu*
Kosho Yamanouchi, *Nagasaki*
Ichiro Yasuda, *Gifu*
Yutaka Yata, *Maebashi-city*
Shin-ichi Yokota, *Sapporo*
Norimasa Yoshida, *Kyoto*
Hiroshi Yoshida, *Tama-City*
Hitoshi Yoshiji, *Kashihara*
Kazuhiko Yoshimatsu, *Tokyo*
Kentaro Yoshioka, *Toyoake*
Nobuhiro Zaima, *Nara*

Jordan

Khaled Ali Jadallah, *Irbid*

Kuwait

Islam Khan, *Kuwait*

Lebanon

Bassam N Abboud, *Beirut*
Kassem A Barada, *Beirut*
Marwan Ghosn, *Beirut*
Iyad A Issa, *Beirut*
Fadi H Mourad, *Beirut*
AIa Sharara, *Beirut*
Rita Slim, *Beirut*

Lithuania

Antanas Mickevicius, *Kaunas*

Malaysia

Huck Joo Tan, *Petaling Jaya*

Mexico

Richard A Awad, *Mexico City*
Carlos R Camara-Lemarroy, *Monterrey*
Norberto C Chavez-Tapia, *Mexico City*
Wolfgang Gaertner, *Mexico City*
Diego Garcia-Compean, *Monterrey*
Arturo Panduro, *Guadalajara*
OT Teramoto-Matsubara, *Mexico City*
Felix Tellez-Avila, *Mexico City*
Omar Vergara-Fernandez, *Mexico City*
Saúl Villa-Trevino, *Cuidad de México*

Morocco

Samir Ahboucha, *Khouribga*

Netherlands

Robert J de Knegt, *Rotterdam*
Tom Johannes Gerardus Gevers, *Nijmegen*
Menno Hoekstra, *Leiden*
BW Marcel Spanier, *Arnhem*
Karel van Erpecum, *Utrecht*

New Zealand

Leo K Cheng, *Auckland*
Andrew Stewart Day, *Christchurch*
Jonathan Barnes Koea, *Auckland*
Max Petrov, *Auckland*

Nigeria

Olufunmilayo Adenike Lesi, *Lagos*
Jesse Abiodun Otegbayo, *Ibadan*
Stella Ifeanyi Smith, *Lagos*

Norway

Trond Berg, *Oslo*
Trond Arnulf Buanes, *Krokkleiva*
Thomas de Lange, *Rud*
Magdy El-Salhy, *Stord*
Rasmus Goll, *Tromso*
Dag Arne Lihaug Hoff, *Aalesund*

Pakistan

Zaigham Abbas, *Karachi*
Usman A Ashfaq, *Faisalabad*
Muhammad Adnan Bawany, *Hyderabad*
Muhammad Idrees, *Lahore*
Saeed Sadiq Hamid, *Karachi*
Yasir Waheed, *Islamabad*

Poland

Thomas Brzozowski, *Cracow*
Magdalena Chmiela, *Lodz*
Krzysztof Jonderko, *Sosnowiec*
Anna Kasicka-Jonderko, *Sosnowiec*
Michal Kukla, *Katowice*
Tomasz Hubert Mach, *Krakow*
Agata Mulak, *Wroclaw*
Danuta Owczarek, *Kraków*
Piotr Socha, *Warsaw*
Piotr Stalke, *Gdansk*
Julian Teodor Swierczynski, *Gdansk*
Anna M Zawilak-Pawlik, *Wroclaw*

Portugal

Marie Isabelle Cremers, *Setubal*
Ceu Figueiredo, *Porto*
Ana Isabel Lopes, *LIsbon*
M Paula Macedo, *Lisboa*
Ricardo Marcos, *Porto*
Rui T Marinho, *Lisboa*
Guida Portela-Gomes, *Estoril*
Filipa F Vale, *Lisbon*

Puerto Rico

Caroline B Appleyard, *Ponce*

Qatar

Abdulbari Bener, *Doha*

Romania

Mihai Ciocirlan, *Bucharest*
Dan LucianDumitrascu, *Cluj-Napoca*
Carmen Fierbinteanu-Braticevici, *Bucharest*
Lucian Negreanu, *Bucharest*
Adrian Saftoiu, *Craiova*
Andrada Seicean, *Cluj-Napoca*
Ioan Sporea, *Timisoara*
Letiția Adela Maria Streba, *Craiova*
Anca Trifan, *Iasi*

Russia

Victor Pasechnikov, *Stavropol*
Vasiliy Ivanovich Reshetnyak, *Moscow*
Vitaly Skoropad, *Obninsk*

Saudi Arabia

Abdul-Wahed N Meshikhes, *Dammam*
M Ezzedien Rabie, *Khamis Mushait*

Singapore

Brian KP Goh, *Singapore*
Richie Soong, *Singapore*
Ker-Kan Tan, *Singapore*
Kok-Yang Tan, *Singapore*
Yee-Joo Tan, *Singapore*
Mark Wong, *Singapore*
Hong Ping Xia, *Singapore*

Slovenia

Matjaz Homan, *Ljubljana*
Martina Perse, *Ljubljana*

South Korea

Sang Hoon Ahn, *Seoul*
Soon Koo Baik, *Wonju*
Soo-Cheon Chae, *Iksan*
Byung-Ho Choe, *Daegu*
Suck Chei Choi, *Iksan*
Hoon Jai Chun, *Seoul*
Yeun-Jun Chung, *Seoul*
Young-Hwa Chung, *Seoul*
Ki-Baik Hahm, *Seongnam*
Sang Young Han, *Busan*

Seok Joo Han, *Seoul*
Seung-Heon Hong, *Iksan*
Jin-Hyeok Hwang, *Seoungnam*
Jeong Won Jang, *Seoul*
Jin-Young Jang, *Seoul*
Dae-Won Jun, *Seoul*
Young Do Jung, *Kwangju*
Gyeong Hoon Kang, *Seoul*
Sung-Bum Kang, *Seoul*
Koo Jeong Kang, *Daegu*
Ki Mun Kang, *Jinju*
Chang Moo Kang, *Seodaemun-gu*
Sang Soo Kim, *Goyang-si*
Jin Cheon Kim, *Seoul*
Tae Il Kim, *Seoul*
Jin Hong Kim, *Suwon*
Kyung Mo Kim, *Seoul*
Kyongmin Kim, *Suwon*
Hyung-Ho Kim, *Seongnam*
Seoung Hoon Kim, *Goyang*
Sang Il Kim, *Seoul*
Hyun-Soo Kim, *Wonju*
Jung Mogg Kim, *Seoul*
Dong Yi Kim, *Gwangju*
Kyun-Hwan Kim, *Seoul*
Jong-Han Kim, *Ansan*
Ja-Lok Ku, *Seoul*
Kyu Taek Lee, *Seoul*
Hae-Wan Lee, *Chuncheon*
Inchul Lee, *Seoul*
Jung Eun Lee, *Seoul*
Sang Chul Lee, *Daejeon*
Song Woo Lee, *Ansan-si*
Hyuk-Joon Lee, *Seoul*
Seong-Wook Lee, *Yongin*
Kil Yeon Lee, *Seoul*
Jong-Inn Lee, *Seoul*
Kyung A Lee, *Seoul*
Jong-Baeck Lim, *Seoul*
Eun-Yi Moon, *Seoul*
SH Noh, *Seoul*
Seung Woon Paik, *Seoul*
Won Sang Park, *Seoul*
Sung-Joo Park, *Iksan*
Kyung Sik Park, *Daegu*
Se Hoon Park, *Seoul*
Yoonkyung Park, *Gwangju*
Seung-Wan Ryu, *Daegu*
Dong Wan Seo, *Seoul*
Il Han Song, *Cheonan*
Myeong Jun Song, *Daejeon*
Yun Kyoung Yim, *Daejeon*
Dae-Yeul Yu *Daejeon*

Spain

Mariam Aguas, *Valencia*
Raul J Andrade, *Málaga*
Antonio Arroyo, *Elche*
Josep M Bordas, *Barcelona*
Lisardo Boscá, *Madrid*
Ricardo Robles Campos, *Murcia*
Jordi Camps, *Reus*
Carlos Cervera *Barcelona*
Alfonso Clemente, *Granada*
Pilar Codoner-Franch, *Valencia*
Fernando J Corrales, *Pamplona*
Fermin Sánchez de Medina, *Granada*
Alberto Herreros de Tejada, *Majadahonda*
Enrique de-Madaria, *Alicante*
JE Dominguez-Munoz, *Santiago de Compostela*
Vicente Felipo, *Valencia*
CM Fernandez-Rodriguez, *Madrid*
Carmen Frontela-Saseta, *Murcia*
Julio Galvez, *Granada*
Maria Teresa García, *Vigo*
MI Garcia-Fernandez, *Málaga*
Emilio Gonzalez-Reimers, *La Laguna*
Marcel Jimenez, *Bellaterra*
Angel Lanas, *Zaragoza*
Juan Ramón Larrubia, *Guadalajara*
Antonio Lopez-Sanroman, *Madrid*
Vicente Lorenzo-Zuniga, *Badalona*
Alfredo J Lucendo, *Tomelloso*
Vicenta Soledad Martinez-Zorzano, *Vigo*
José Manuel Martin-Villa, *Madrid*
Julio Mayol, *Madrid*
Manuel Morales-Ruiz, *Barcelona*
Alfredo Moreno-Egea, *Murcia*
Albert Pares, *Barcelona*
Maria Pellise, *Barcelona*
José Perea, *Madrid*
Miguel Angel Plaza, *Zaragoza*
María J Pozo, *Cáceres*
Enrique Quintero, *La Laguna*
Jose M Ramia, *Madrid*
Francisco Rodriguez-Frias, *Barcelona*
Silvia Ruiz-Gaspa, *Barcelona*
Xavier Serra-Aracil, *Barcelona*
Vincent Soriano, *Madrid*
Javier Suarez, *Pamplona*
Carlos Taxonera, *Madrid*
M Isabel Torres, *Jaén*
Manuel Vazquez-Carrera, *Barcelona*
Benito Velayos, *Valladolid*
Silvia Vidal, *Barcelona*

Sri Lanka

Arjuna Priyadarsin De Silva, *Colombo*

Sudan

Ishag Adam, *Khartoum*

Sweden

Roland G Andersson, *Lund*
Bergthor Björnsson, *Linkoping*
Johan Christopher Bohr, *Örebro*
Mauro D'Amato, *Stockholm*
Thomas Franzen, *Norrkoping*
Evangelos Kalaitzakis, *Lund*
Riadh Sadik, *Gothenburg*
Per Anders Sandstrom, *Linkoping*
Ervin Toth, *Malmö*
Konstantinos Tsimogiannis, *Vasteras*
Apostolos V Tsolakis, *Uppsala*

Switzerland

Gieri Cathomas, *Liestal*
Jean Louis Frossard, *Geneve*
Christian Toso, *Geneva*
Stephan Robert Vavricka, *Zurich*
Dominique Velin, *Lausanne*

Thailand

Thawatchai Akaraviputh, *Bangkok*
P Yoysungnoen Chintana, *Pathumthani*
Veerapol Kukongviriyapan, *Muang*
Vijittra Leardkamolkarn, *Bangkok*
Varut Lohsiriwat, *Bangkok*
Somchai Pinlaor, *Khaon Kaen*
D Wattanasirichaigoon, *Bangkok*

Trinidad and Tobago

B Shivananda Nayak, *Mount Hope*

Tunisia

Ibtissem Ghedira, *Sousse*
Lilia Zouiten-Mekki, *Tunis*

Turkey

Sami Akbulut, *Diyarbakir*
Inci Alican, *Istanbul*
Mustafa Altindis, *Sakarya*
Mutay Aslan, *Antalya*
Oktar Asoglu, *Istanbul*
Yasemin Hatice Balaban, *Istanbul*
Metin Basaranoglu, *Ankara*
Yusuf Bayraktar, *Ankara*
Süleyman Bayram, *Adiyaman*
Ahmet Bilici, *Istanbul*
Ahmet Sedat Boyacioglu, *Ankara*
Züleyha Akkan Cetinkaya, *Kocaeli*
Cavit Col, *Bolu*
Yasar Colak, *Istanbul*
Cagatay Erden Daphan, *Kirikkale*
Mehmet Demir, *Hatay*
Ahmet Merih Dobrucali, *Istanbul*
Gülsüm Ozlem Elpek, *Antalya*
Ayse Basak Engin, *Ankara*
Eren Ersoy, *Ankara*
Osman Ersoy, *Ankara*
Yusuf Ziya Erzin, *Istanbul*
Mukaddes Esrefoglu, *Istanbul*
Levent Filik, *Ankara*
Ozgur Harmanci, *Ankara*
Koray Hekimoglu, *Ankara*
Abdurrahman Kadayifci, *Gaziantep*
Cem Kalayci, *Istanbul*
Selin Kapan, *Istanbul*
Huseyin Kayadibi, *Adana*
Sabahattin Kaymakoglu, *Istanbul*
Metin Kement, *Istanbul*
Mevlut Kurt, *Bolu*
Resat Ozaras, *Istanbul*
Elvan Ozbek, *Adapazari*
Cengiz Ozcan, *Mersin*
Hasan Ozen, *Ankara*
Halil Ozguc, *Bursa*
Mehmet Ozturk, *Izmir*
Orhan V Ozkan, *Sakarya*
Semra Paydas, *Adana*
Ozlem Durmaz Suoglu, *Istanbul*
Ilker Tasci, *Ankara*

Müge Tecder-ünal, *Ankara*
Mesut Tez, *Ankara*
Serdar Topaloglu, *Trabzon*
Murat Toruner, *Ankara*
Gokhan Tumgor, *Adana*
Oguz Uskudar, *Adana*
Mehmet Yalniz, *Elazig*
Mehmet Yaman, *Elazig*
Veli Yazisiz, *Antalya*
Yusuf Yilmaz, *Istanbul*
Ozlem Yilmaz, *Izmir*
Oya Yucel, *Istanbul*
Ilhami Yuksel, *Ankara*

United Kingdom

Nadeem Ahmad Afzal, *Southampton*
Navneet K Ahluwalia, *Stockport*
Yeng S Ang, *Lancashire*
Ramesh P Arasaradnam, *Coventry*
John Beynon, *Swansea*
Barbara Braden, *Oxford*
Simon Bramhall, *Birmingham*
Geoffrey Burnstock, *London*
Ian Chau, *Sutton*
Thean Soon Chew, *London*
Helen G Coleman, *Belfast*
Anil Dhawan, *London*
Sunil Dolwani, *Cardiff*
Piers Gatenby, *London*
Anil T George, *London*
Pasquale Giordano, *London*
Paul Henderson, *Edinburgh*
Georgina Louise Hold, *Aberdeen*
Stefan Hubscher, *Birmingham*
Robin D Hughes, *London*
Nusrat Husain, *Manchester*
Matt W Johnson, *Luton*
Konrad Koss, *Macclesfield*
Anastasios Koulaouzidis, *Edinburgh*
Simon Lal, *Salford*
John S Leeds, *Aberdeen*
Hongxiang Liu, *Cambridge*
Michael Newton Marsh, *Oxford*
Michael Joseph McGarvey, *London*
Michael Anthony Mendall, *London*
Alexander H Mirnezami, *Southampton*
J Bernadette Moore, *Guildford*
Claudio Nicoletti, *Norwich*
Savvas Papagrigoriadis, *London*
David Mark Pritchard, *Liverpool*
James A Ross, *Edinburgh*
Kamran Rostami, *Worcester*
Xiong Z Ruan, *London*
Dina Tiniakos, *Newcastle upon Tyne*
Frank I Tovey, *London*
Dhiraj Tripathi, *Birmingham*
Vamsi R Velchuru, *Great Yarmouth*
Nicholas T Ventham, *Edinburgh*
Diego Vergani, *London*
Jack Westwood Winter, *Glasgow*
Terence Wong, *London*
Ling Yang, *Oxford*

United States

Daniel E Abbott, *Cincinnati*
Ghassan K Abou-Alfa, *New York*
Julian Abrams, *New York*
David William Adelson, *Los Angeles*
Jonathan Steven Alexander, *Shreveport*
Tauseef Ali, *Oklahoma City*
Mohamed R Ali, *Sacramento*
Rajagopal N Aravalli, *Minneapolis*
Hassan Ashktorab, *Washington*
Shashi Bala, *Worcester*
Charles F Barish, *Raleigh*
P Patrick Basu, *New York*
Robert L Bell, *Berkeley Heights*
David Bentrem, *Chicago*
Henry J Binder, *New Haven*
Joshua Bleier, *Philadelphia*
Wojciech Blonski, *Johnson City*
Kenneth Boorom, *Corvallis*
Brian Boulay, *Chicago*
Carla W Brady, *Durham*
Kyle E Brown, *Iowa City*
Adeel AButt, *Pittsburgh*
Weibiao Cao, *Providence*
Andrea Castillo, *Cheney*
Fernando J Castro, *Weston*
Adam S Cheifetz, *Boston*
Adam S Cheifetz, *Boston*
Xiaoxin Luke Chen, *Durham*
Ramsey Cheung, *Palo Alto*
Parimal Chowdhury, *Little Rock*
Edward John Ciaccio, *New York*
Dahn L Clemens, *Omaha*
Yingzi Cong, *Galveston*
Laura Iris Cosen-Binker, *Boston*
Joseph John Cullen, *Lowa*
Mark J Czaja, *Bronx*
Mariana D Dabeva, *Bronx*
Christopher James Damman, *Seattle*
Isabelle G De Plaen, *Chicago*
Abhishek Deshpande, *Cleveland*
Punita Dhawan, *Nashville*
Hui Dong, *La Jolla*
Wael El-Rifai, *Nashville*
Sukru H Emre, *New Haven*
Paul Feuerstadt, *Hamden*
Josef E Fischer, *Boston*
Laurie N Fishman, *Boston*
Temitope Foster, *Atlanta*
AmyEFoxx-Orenstein, *Scottsdale*
Daniel E Freedberg, *New York*
Shai Friedland, *Palo Alto*
Virgilio George, *Indianapolis*
Ajay Goel, *Dallas*
Oliver Grundmann, *Gainesville*
Stefano Guandalini, *Chicago*
Chakshu Gupta, *St. Joseph*
Grigoriy E Gurvits, *New York*
Xiaonan Han, *Cincinnati*
Mohamed Hassan, *Jackson*
Martin Hauer-Jensen, *Little Rock*
Koichi Hayano, *Boston*
Yingli Hee, *Atlanta*
Samuel B Ho, *San Diego*
Jason Ken Hou, *Houston*
Lifang Hou, *Chicago*
K-Qin Hu, *Orange*
Jamal A Ibdah, *Columbia*
Robert Thomas Jensen, *Bethesda*
Huanguang "Charlie" Jia, *Gainesville*
Rome Jutabha, *Los Angeles*
Andreas M Kaiser, *Los Angeles*
Avinash Kambadakone, *Boston*
David Edward Kaplan, *Philadelphia*
Randeep Kashyap, *Rochester*
Rashmi Kaul, *Tulsa*
Ali Keshavarzian, *Chicago*
Amir Maqbul Khan, *Marshall*
Nabeel Hasan Khan, *New Orleans*
Sahil Khanna, *Rochester*
Kusum K Kharbanda, *Omaha*
Hyun Sik Kim, *Pittsburgh*
Joseph Kim, *Duarte*
Jae S Kim, *Gainesville*
Miran Kim, *Providence*
Timothy R Koch, *Washington*
Burton I Korelitz, *New York*
Betsy Kren, *Minneapolis*
Shiu-Ming Kuo, *Buffalo*
Michelle Lai, *Boston*
Andreas Larentzakis, *Boston*
Edward Wolfgang Lee, *Los Angeles*
Daniel A Leffler, *Boston*
Michael Leitman, *New York*
Suthat Liangpunsakul, *Indianapolis*
Joseph K Lim, *New Haven*
Elaine Y Lin, *Bronx*
Henry C Lin, *Albuquerque*
Rohit Loomba, *La Jolla*
James David Luketich, *Pittsburgh*
Mohammad F Madhoun, *Oklahoma City*
Thomas C Mahl, *Buffalo*
Ashish Malhotra, *Bettendorf*
Pranoti Mandrekar, *Worcester*
John Marks, *Wynnewood*
Wendy M Mars, *Pittsburgh*
Julien Vahe Matricon, *San Antonio*
Craig J McClain, *Louisville*
George K Michalopoulos, *Pittsburgh*
Tamir Miloh, *Phoenix*
Ayse Leyla Mindikoglu, *Baltimore*
Huanbiao Mo, *Denton*
Klaus Monkemuller, *Birmingham*
John Morton, *Stanford*
Adnan Muhammad, *Tampa*
Michael J Nowicki, *Jackson*
Patrick I Okolo, *Baltimore*
Giusepp Orlando, *Winston Salem*
Natalia A Osna, *Omaha*
Virendra N Pandey, *Newark*
Mansour A Parsi, *Cleveland*
Michael F Picco, *Jacksonville*
Daniel S Pratt, *Boston*
Xiaofa Qin, *Newark*
Janardan K Reddy, *Chicago*
Victor E Reyes, *Galveston*
Jon Marc Rhoads, *Houston*
Giulia Roda, *New York*
Jean-Francois Armand Rossignol, *Tampa*
Paul A Rufo, *Boston*
Madhusudana Girija Sanal, *New York*
Miguel Saps, *Chicago*
Sushil Sarna, *Galveston*
Ann O Scheimann, *Baltimore*
Bernd Schnabl, *La Jolla*
Matthew J Schuchert, *Pittsburgh*
Ekihiro Seki, *La Jolla*
Chanjuan Shi, *Nashville*
David Quan Shih, *Los Angeles*
William B Silverman, *Iowa City*
Shashideep Singhal, *New York*
Bronislaw L Slomiany, *Newark*
Steven F Solga, *Bethlehem*
Byoung-Joon Song, *Bethesda*
Dario Sorrentino, *Roanoke*
Scott R Steele, *Fort Lewis*

World Journal of Gastroenterology

Contents

Weekly Volume 20 Number 4 January 28, 2014

TOPIC HIGHLIGHT

Contents

World Journal of Gastroenterology
Volume 20 Number 4 January 28, 2014

AIMS AND SCOPE

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1339 experts in gastroenterology and hepatology from 67 countries.

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

INDEXING/ABSTRACTING

World Journal of Gastroenterology is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. ISI, Journal Citation Reports®, Gastroenterology and Hepatology, 2012 Impact Factor: 2.547 (34/74); Total Cites: 19145 (6/74); Current Articles: 944 (1/74); and Eigenfactor® Score: 0.06035 (6/74).

NAME OF JOURNAL
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

LAUNCH DATE
October 1, 1995

FREQUENCY
Weekly

EDITORIAL OFFICE
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: bpgoffice@wjgnet.com
http://www.wjgnet.com

PUBLISHER
Baishideng Publishing Group Co., Limited
Flat C, 23/F, Lucky Plaza,
315-321 Lockhart Road, Wan Chai, Hong Kong, China
Fax: +852-65557188
Telephone: +852-31779906
E-mail: bpgoffice@wjgnet.com
http://www.wjgnet.com

PUBLICATION DATE
January 28, 2014

INSTRUCTIONS TO AUTHORS
Full instructions are available online at http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm

ONLINE SUBMISSION
http://www.wjgnet.com/esps/

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.869

World J Gastroenterol 2014 January 28; 20(4): 869-876
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Colorectal cancer disparities: Issues, controversies and solutions

Venkata S Tammana, Adeyinka O Laiyemo

Venkata S Tammana, Adeyinka O Laiyemo, Division of Gastroenterology, Department of Medicine, Howard University College of Medicine, Washington, DC 20060, United States
Author contributions: Laiyemo AO designed the outline of the article; Tammana VS and Laiyemo AO have contributed equally in collecting the data, drafting of the manuscript and preparing the final manuscript.
Correspondence to: Adeyinka O Laiyemo, MD, MPH, Division of Gastroenterology, Department of Medicine, Howard University College of Medicine, 2041 Georgia Avenue, Washington, DC 20060, United States. adeyinka.laiyemo@howard.edu
Telephone: +1-202-8657186 Fax: +1-202-8656562
Received: October 5, 2013 Revised: November 14, 2013
Accepted: December 5, 2013
Published online: January 28, 2014

Core tip: This article reviews the underlying factors for the disproportionally higher burden of colorectal cancer (CRC) among blacks, addresses controversies regarding race-based screening recommendations and concludes by suggesting that a comprehensive approach that increases access and utilization of CRC screening, timely follow-up of abnormal results and treatment of CRC will be needed to reduce or eliminate CRC disparity.

Tammana VS, Laiyemo AO. Colorectal cancer disparities: Issues, controversies and solutions. *World J Gastroenterol* 2014; 20(4): 869-876 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/869.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.869

Abstract

Colorectal cancer (CRC) is the second leading cause of cancer related deaths in the United States. There are significant differences in CRC incidence and mortality by race with the highest burden occurring among blacks. The underlying factors contributing to CRC disparities are multiple and complex. Studies have suggested that a higher prevalence of putative risk factors for CRC, limited access to healthcare services, lower utilization of healthcare resources and increased biological susceptibilities contribute to this disparity by race. This article reviews the factors associated with the disproportionally higher burden of CRC among blacks; addresses the controversies regarding the age to begin CRC screening and the screening modality to use for blacks; and proffers solutions to eliminate CRC disparity by race.

Key words: Colorectal cancer disparities; Adenomatous polyps; Colon cancer; Colonoscopy; Screening

DISPARITIES IN COLORECTAL CANCER INCIDENCE AND MORTALITY BY RACE

Widening racial disparities in cancer burden continues to be recognized[1-5]. Since 1960, colorectal cancer (CRC) mortality has declined by 39% among whites, but increased by 28% among blacks[5]. The incidence and mortality from CRC is higher among blacks when compared with other race-ethnicities[2]. An estimated 142820 new cases of CRC and 50830 CRC related deaths were expected in 2013. The incidence of CRC among black males is 65.1 per 100000 population but 52.8 per 100000 among white males. The incidence of CRC among black females is 48 per 100000 as compared to 39.2 per 100000 among white females. Similar pattern has been observed with mortality rates from the disease. The CRC mortality rate among black males is 29.8 per 100000 as compared to 19.5 per 100000 among white males. The mortality rate from CRC is 19.8 per 100000 among black females but 13.6 per 100000 among white females.

POSTULATED FACTORS UNDERLYING RACIAL DISPARITY IN CRC BURDEN

Differences in the prevalence of putative diet and lifestyle risk factors

Studies have suggested that alcohol ingestion, cigarette smoking, obesity, and meat consumption increases the risk of CRC whereas physical activity decreases the risk[6,7]. The prevalence of obesity and cigarette smoking is higher and physical activity is lower among blacks when compared to whites[8,9]. In South Africa, blacks have lower incidence of CRC (< 5 per 100000) as compared to South African whites (40 per 100000) who consumed a typical "western" diet[10]. When compared to South African blacks, American blacks exhibit a higher CRC risk and mucosal proliferation rates which is associated with higher dietary intakes of animal products and higher colonic populations of potentially toxic hydrogen and secondary bile-salt-producing bacteria[10]. Higher consumption of animal fat including beef, pork and lamb has been associated with an increased risk for CRC[11,12]. The consumption of red meat and pork is higher among blacks than other race-ethnicities in the United States[12].

Some studies have suggested an inverse association between micronutrients (Vitamin E, β carotene, vitamin C, Calcium) intake and CRC risk[13,14]. However, there are differences in the intake of micronutrients between blacks and whites with higher intake among whites primarily due to higher dietary supplements use. A possible basis of this association is that high concentrations of hydrogen (a byproduct of bacterial metabolism) in the colon can interfere with cellular metabolism[15]. Hydrogen concentration has been reported to be higher among blacks than among whites[10] and hydrogen is toxic to colonic epithelium. Although the exact mechanisms of action of micronutrients are not well established, they are postulated to have anti-oxidant effects, exert some effect on cell growth regulation and enhance immune response.

Although these dietary and lifestyle factors have been associated with CRC risk and may play a role in the observed disparity among blacks versus whites, the actual differential contributions of these factors to the overall CRC burden is not well established. For instance, while it has been suggested that high fat and low fiber diet are associated with increased susceptibility to CRC, reversal or adoption of the "healthy" lifestyle has not been proven to reduce CRC susceptibility in randomized trials. For instance, the adoption of low fat, high fiber, high fruits and vegetable diet in the Polyp Prevention Trial and fiber supplementation in the Wheat Bran Fiber Trial did not reduce the recurrence of adenoma[16,17]. Similarly, weight loss within 4 years did not reduce the risk of adenoma recurrence[18].

Furthermore, using the Behavioral Risk Factor Surveillance System, the prevalence of these putative risk factors such as obesity, cigarette smoking, alcohol consumption, lack of physical activities, low socioeconomic status and low health literacy are comparable among blacks and Hispanic population, yet Hispanic Americans have lower burden of CRC when compared to blacks and whites in the United States[2,9]. The incidence of CRC among Hispanic males and Hispanic females are 46.9 and 33.3 per 100000 population and the corresponding mortality rates are 15.3 and 10.2 per 100000 population.

Differences in tumor biology

Blacks tend to be diagnosed with CRC at younger ages, are more likely to present with proximal tumors and be diagnosed with more advanced diseases than whites[19-26]. This suggests that stage at presentation may account for some of the observed racial differences in survival after CRC diagnosis. Tumor grade is an independent prognostic predictor of CRC even after adjusting the effects of stage of disease at the time of diagnosis[3,27-31]. Studies have suggested that blacks suffer worse outcomes when they present with similar tumor characteristics as whites. Alexander *et al*[31] reported that blacks with high grade tumors were three times (HR = 3.05, 95%CI: 1.32-7.05) more likely to die of CRC within 5 years after surgery when compared with whites with high grade tumors. Blacks with lymph node-negative CRC have more than 40% excess mortality when compared to whites with the same disease[5,26,27]. It is noteworthy that approximately 30% of patients with lymph node negative CRC (stages Ⅰ and Ⅱ) develop recurrent disease, probably reflecting the presence of occult tumor in those lymph nodes[32-34]. Hyslop *et al*[35] suggested that blacks exhibited 4-fold greater occult metastases in individual lymph nodes compared with whites. Thus, occult tumor burden may be playing an important role in racial disparities in CRC outcomes between blacks and whites. However, in a systematic review of published studies, Bach *et al*[36] reported that there were no appreciable differences in cancer-specific survival between blacks and whites when treatment was comparable for similar stage cancers. The authors concluded that differences in cancer biology between racial groups are unlikely to be responsible for a substantial portion of the observed discrepancy in stage-specific CRC survival if blacks and whites receive similar treatments.

Differences in healthcare access, utilization and treatment

Blacks are more likely to be uninsured, have a fatalistic attitude towards medical illness, experience stigma, exhibit fear and denial related to a cancer diagnosis, have an aversion to health care treatments such as surgery, mistrust the healthcare system, and have misperceptions about cancer that ultimately interfere with treatment[9,37,38]. Although the efficacy of treatment for CRC appeared to be similar between blacks and whites in equal access systems and among participants in adjuvant chemotherapy trials[21,23] there are differences in the treatment received after CRC diagnosis among blacks and whites in general[25,26,39-45].

Blacks received surgery less often than whites for CRC[26,42-45]. Demissie *et al*[44] reported that the odds of non-receipt of surgical treatment was higher among

blacks as compared with whites for stage I (OR = 2.08, 95%CI: 1.41-3.03 among males; OR = 2.38; 95%CI: 1.69-3.45 among females) and stage Ⅳ colon cancer (OR = 1.25, 95%CI: 1.01-1.56 among males; OR = 1.41, 95%CI: 1.14-1.72 among females). Similar results were reported by Le *et al*[26]. The authors reported that a higher percentage of blacks did not undergo surgical resection for stage I disease (5.4% *vs* 3.7%, $P < 0.001$) and stage Ⅳ disease (30.3% *vs* 23.3%, $P < 0.001$) when compared with whites. In general, the reasons for not receiving surgery among blacks included that it was less often recommended, refused by black patients, and because of higher prevalence of comorbid conditions among blacks[26].

While disparity in surgical treatment of CRC between blacks and whites is evident from above, studies have shown mixed results regarding chemotherapy and radiation therapy use for CRC management by race. Some studies suggest that blacks were less likely to undergo chemotherapy and radiation therapy even when they had undergone consultations with oncologists[25,40,46]. The reason for this is unclear but it is unknown if blacks are more fearful of the effects of chemotherapy even though blacks were less likely to develop chemotherapy related toxicity in a clinical trial[47]. However, it has been suggested that a higher proportion of black patients with CRC lived in census tracts with low high school graduation rates and these areas had lower chemotherapy use[48]. Blacks also tend to have less supplemental coverage when compared to whites which could affect co-payment for outpatient chemotherapy, and this can affect the initiation of chemotherapy among blacks[41]. Conversely, other studies did not find an association between race and chemotherapy use[26,49]. It is noteworthy that studies have reported that disparity in survival after CRC diagnosis are attenuated or eliminated with the receipt of similar treatments by blacks and whites[21,23,47,50,51].

Disparities in colorectal cancer screening

There has been growing evidence that CRC screening reduces mortality[52-54]. There are multiple acceptable options for CRC screening including fecal occult blood and fecal DNA testing, double contrast barium enema, CT colonography, flexible sigmoidoscopy, and colonoscopy[55]. Identified significant predictors of CRC screening include older age, having a regular doctor and participating in general medical examinations[56]. Screening rates are low among racial and ethnic minorities and persons from socioeconomically disadvantaged population[57-59]. Doubeni *et al*[60] reported that the screening rate for white Medicare beneficiaries improved from 49% in 2000 to 56.6% in 2005. During the same study period, the screening rates among blacks improved from 41% to 52% suggesting that screening rates for CRC are improving, but disparity in the screening rates still persists. Tehranifar *et al*[61] also reported that cancer survival disparities between blacks and whites widened as cancers become more amenable to medical interventions. This suggests that blacks lag behind when medical technologies that improve cancer healthcare are introduced for reasons that are not well understood.

Disparities in screening have been cited as one of the reasons for high incidence and mortality from CRC among blacks as compared with whites. Lansdorp-Vogelaar *et al*[62] reported that differences in CRC screening accounted for 42% of disparity in CRC incidence and 19% of disparity in CRC mortality between blacks and whites.

There are many barriers to CRC screening uptake among blacks. These are due to a complex combination of socioeconomic disadvantages from lower education and income, place of residence, inadequate insurance and mistrust of the medical system[63-65]. People in lower socioeconomic neighborhoods are less likely to undergo a colonoscopy, even among insured subjects receiving care in integrated healthcare systems[64]. Physician recommendation is important for completing CRC screening[66,67]. However, increasing availability of primary care physicians and colonoscopy providers did not narrow the racial gaps in CRC screening disparities. Rather, colonoscopy rates increased among whites but decreased among minorities[59]. Therefore, there is an urgent need to increase the participation of minorities as care providers in biomedical fields and improve cultural competencies of all care providers[68,69].

CONTROVERSIES IN COLORECTAL CANCER SCREENING BY RACE

Although it is well known that blacks suffer the highest burden from CRC and increasing CRC screening uptake among blacks is at the core of the solution, there is no consensus regarding the screening strategy to adopt in eliminating this disparity. In particular, it is unclear whether there should be different CRC screening recommendations based on race-ethnicity.

Should we begin CRC screening earlier among blacks?

Studies have suggested that when compared to whites, blacks are more likely to be diagnosed with CRC at younger ages, present with proximal tumors and be diagnosed with advanced diseases[19-26]. The widely adopted recommendation is to begin CRC screening from 50 years of age for average risk individuals in the United States[55,70]. Some experts have expressed the opinion that race-based recommendation for CRC screening will create more confusion for patients and their healthcare providers amidst a crowded field of recommendations based on age, family history of colorectal cancer and polyps, and colonic diseases such as inflammatory bowel disease. They argue for increasing efforts to boost participation in CRC screening by blacks and that it is not cost effective to lower the age of screening[71]. In contrast to this opinion, the American College of Physicians recommended that CRC screening should start at 40 years of age[72] while the American College of Gastroenterology (ACG)[73] and American Society for Gastrointestinal Endoscopy recommended CRC screening beginning at age

45 years[74]. In our opinion, blacks should begin screening at age 45 years as part of a comprehensive intervention to improve screening uptake, ensure adequate follow-up and resolution of abnormal screening results, and enhance access and utilization of good quality treatment of patients diagnosed with CRC in a timely fashion. This is because of the well documented earlier presentation with CRC among blacks and the proximal location of their tumors which raises the possibility of genetic predisposition such as Lynch syndrome. However, genetic predisposition to CRC is raised based on family history of CRC. It is well known that blacks tend not to know their family medical history due to lower health literacy and lack of knowledge of their family ancestry. There is a lack of information among African Americans with regards to the prevalence of inherited conditions that predispose to CRC. Murff *et al*[75] reported that blacks who have first-degree relatives (FDRs) with CRC are less likely to undergo colonoscopy screening compared with whites (27.3% *vs* 43.1%) who have affected relatives. Therefore, screening blacks earlier will serve as a "reset" that has the potential of early identification of those at increased risk of CRC in the next generation. Future studies should focus on quantifying genetic susceptibility to CRC among blacks.

What CRC screening modality should be recommended for blacks?

The predisposition to proximal CRC among blacks is a potent argument to support the recommendation of the ACG that colonoscopy should be the preferred screening modality. Furthermore, colonoscopy is the diagnostic procedure after an abnormal screening from the other modalities and polyps can be removed during the same procedure. However, it is the most invasive, most expensive and presents the most difficult logistic challenge in terms of bowel preparation, co-pays, missed work days and the need for an escort due to moderate sedation. Studies have suggested a higher completion rate of screening when the underserved are offered fecal occult blood tests[76,77]. It appears that there are wide geographic variations in availability of colonoscopy to the population and in the acceptability of fecal based tests to the underserved. We observed that blacks were less likely to undergo diagnostic colonoscopy following an abnormal flexible sigmoidoscopy screening in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial[78]. This raises a concern that follow-up of abnormal screening may be a challenge for blacks and a screening modality that can permit intervention may be better. Notwithstanding, we are of the opinion that healthcare providers should discuss CRC screening options with their patients, but should make a specific recommendation for a screening modality based on the patient's preferences, comorbidities, social situation, and locally available resources.

THE WAY FORWARD

It will require a comprehensive patient, provider, and policy-maker partnership to reduce CRC disparity. As studies have shown that blacks who receive medical care and cancer treatment similar to those of whites experience similar outcomes, enabling blacks to achieve equal access to care as whites could substantially reduce the racial disparities in CRC burden[36]. It is imperative to improve health insurance coverage and health education of the population. The implementation of the Affordable Care Act has a potential to ensure coverage for millions of previously uninsured persons and provide the necessary access to CRC screening. However, White *et al*[79] reported that despite the expansion of Medicare coverage for CRC screening tests, racial/ethnic differences in CRC screening persisted over time among this insured population. Hence, health care access by provision of health insurance is necessary but not sufficient to improve CRC screening and reduce CRC disparity by race.

In a meta analysis by Naylor *et al*[80] provider-directed multi-modal interventions which comprised of education sessions and reminders and pure educational interventions were found to be effective in raising CRC screening rates in minorities by 10%-15%. A median improvement of 16% in endoscopic CRC screening completion was noted with the patient navigator model. Tailored patient education combined with patient navigation services, and physician training in communicating with patients of low health literacy, can modestly improve adherence to CRC screening.

The New York City Department of Health and Mental Hygiene has started a program called The Citywide Colon Cancer Control Coalition (C5) to help New York City attain CRC control goals through advocacy, resource development, and policy initiatives. They launched public campaigns and implemented patient navigation systems in many of the New York City area hospitals in an effort to increase colonoscopy screening rates and reduce racial/ethnic disparities. In 2003, low rates of screening as well as screening disparities were noted with only 36% of blacks, 38% Latinos and 48% of whites having had a colonoscopy. After this concerted citywide effort, screening colonoscopy rates had increased to approximately 62 percent by 2009 with virtual elimination of screening disparities among these ethnic groups[81].

In Delaware, a "Village approach" was implemented which involved a 3-step process to reduce CRC disparities by increasing CRC screening rates, providing quality treatment and resolution of abnormalities and by using an extensive patient navigation services. The program led to increased CRC screening rates among all race-ethnicities, reduction in CRC incidence, reduction in CRC mortality and narrowing of the CRC survival differences[82].

Figure 1 shows the leading causes of CRC disparities and proffer solutions to reduce the observed disparities.

CONCLUSION

There are several important factors contributing to differential CRC mortality rates among blacks and whites. A comprehensive approach that increases access and utilization of CRC screening, timely follow-up of abnormal

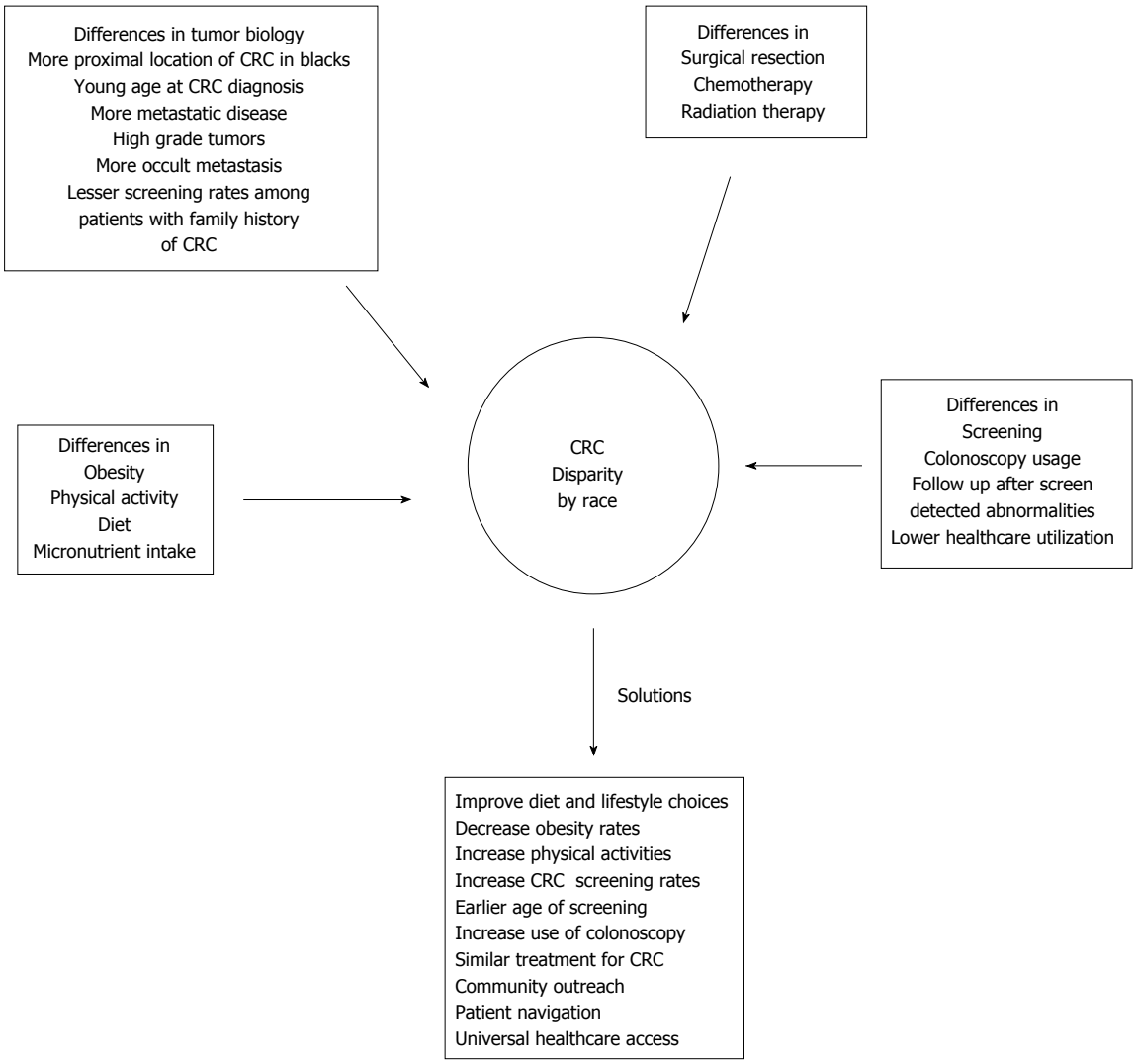


Figure 1 Leading causes of colorectal cancer disparities and potential solutions to reduce the observed disparities. CRC: Colorectal cancer.

results and treatment of CRC will be needed to reduce or eliminate disparity.

REFERENCES

1 **Henschke UK**, Leffall LD, Mason CH, Reinhold AW, Schneider RL, White JE. Alarming increase of the cancer mortality in the U.S. black population (1950-1967). *Cancer* 1973; **31**: 763-768 [PMID: 4706044 DOI: 10.1002/1097-0142(197304)31]

2 **Siegel R**, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin* 2013; **63**: 11-30 [PMID: 23335087 DOI: 10.3322/caac.21166]

3 **Mayberry RM**, Coates RJ, Hill HA, Click LA, Chen VW, Austin DF, Redmond CK, Fenoglio-Preiser CM, Hunter CP, Haynes MA. Determinants of black/white differences in colon cancer survival. *J Natl Cancer Inst* 1995; **87**: 1686-1693 [PMID: 7473817]

4 **Schroy PC**, Glick JT, Robinson PA, Lydotes MA, Evans SR, Emmons KM. Has the surge in media attention increased public awareness about colorectal cancer and screening? *J Community Health* 2008; **33**: 1-9 [PMID: 18080203]

5 **Soneji S**, Iyer SS, Armstrong K, Asch DA. Racial disparities in stage-specific colorectal cancer mortality: 1960-2005. *Am J Public Health* 2010; **100**: 1912-1916 [PMID: 20724684 DOI: 10.2105/AJPH.2009.184192]

6 **Huxley RR**, Ansary-Moghaddam A, Clifton P, Czernichow S, Parr CL, Woodward M. The impact of dietary and lifestyle risk factors on risk of colorectal cancer: a quantitative overview of the epidemiological evidence. *Int J Cancer* 2009; **125**: 171-180 [PMID: 19350627 DOI: 10.1002/ijc.24343]

7 **Ford ES**. Body mass index and colon cancer in a national sample of adult US men and women. *Am J Epidemiol* 1999; **150**: 390-398 [PMID: 10453815]

8 **National Cancer Institute**. Cancer Trends Progress Report 2011-2012. Last accessed on October 1, 2013. Available from: URL: http//progressreport.cancer.gov/doc.asp?

9 **Bolen JC**, Rhodes L, Powell-Griner EE, Bland SD, Holtzman D. State-specific prevalence of selected health behaviors, by race and ethnicity--Behavioral Risk Factor Surveillance System, 1997. *MMWR CDC Surveill Summ* 2000; **49**: 1-60 [PMID: 10965781]

10 **O'Keefe SJ**, Chung D, Mahmoud N, Sepulveda AR, Manafe M, Arch J, Adada H, van der Merwe T. Why do African Americans get more colon cancer than Native Africans? *J Nutr* 2007; **137**: 175S-182S [PMID: 17182822]

11 **Willett WC**, Stampfer MJ, Colditz GA, Rosner BA, Speizer FE. Relation of meat, fat, and fiber intake to the risk of colon cancer in a prospective study among women. *N Engl J Med* 1990; **323**: 1664-1672 [PMID: 2172820 DOI: 10.1056/NEJM199012133232404]

12 **USDA Economic Research Service**. 1994–1996 and 1998

Continuing Survey of Food Intakes by Individuals. 2000. Available from: URL: http//www.ers.usda.gov/publications/ldp/Oct05/ldpm13502/ldpm13502.pdf
13 **Patterson RE**, White E, Kristal AR, Neuhouser ML, Potter JD. Vitamin supplements and cancer risk: the epidemiologic evidence. *Cancer Causes Control* 1997; **8**: 786-802 [PMID: 9328202 DOI: 10.1023/A: 1018443724293]
14 **Satia-Abouta J**, Galanko JA, Martin CF, Potter JD, Ammerman A, Sandler RS. Associations of micronutrients with colon cancer risk in African Americans and whites: results from the North Carolina Colon Cancer Study. *Cancer Epidemiol Biomarkers Prev* 2003; **12**: 747-754 [PMID: 12917206]
15 **Gibson GR**, Macfarlane GT, Cummings JH. Sulphate reducing bacteria and hydrogen metabolism in the human large intestine. *Gut* 1993; **34**: 437-439 [PMID: 8491386 DOI: 10.1136/gut.34.4.437]
16 **Schatzkin A**, Lanza E, Corle D, Lance P, Iber F, Caan B, Shike M, Weissfeld J, Burt R, Cooper MR, Kikendall JW, Cahill J. Lack of effect of a low-fat, high-fiber diet on the recurrence of colorectal adenomas. Polyp Prevention Trial Study Group. *N Engl J Med* 2000; **342**: 1149-1155 [PMID: 10770979 DOI: 10.1056/NEJM200004203421601]
17 **Alberts DS**, Martínez ME, Roe DJ, Guillén-Rodríguez JM, Marshall JR, van Leeuwen JB, Reid ME, Ritenbaugh C, Vargas PA, Bhattacharyya AB, Earnest DL, Sampliner RE. Lack of effect of a high-fiber cereal supplement on the recurrence of colorectal adenomas. Phoenix Colon Cancer Prevention Physicians' Network. *N Engl J Med* 2000; **342**: 1156-1162 [PMID: 10770980 DOI: 10.1056/NEJM200004203421602]
18 **Laiyemo AO**, Doubeni C, Badurdeen DS, Murphy G, Marcus PM, Schoen RE, Lanza E, Smoot DT, Cross AJ. Obesity, weight change, and risk of adenoma recurrence: a prospective trial. *Endoscopy* 2012; **44**: 813-818 [PMID: 22926666 DOI: 10.1055/s-0032-1309837]
19 **Jessup JM**, McGinnis LS, Steele GD, Menck HR, Winchester DP. The National Cancer Data Base. Report on colon cancer. *Cancer* 1996; **78**: 918-926 [PMID: 8756390 DOI: 10.1002/(SICI)1097-0142(19960815)78]
20 **Shavers VL**. Racial/ethnic variation in the anatomic subsite location of in situ and invasive cancers of the colon. *J Natl Med Assoc* 2007; **99**: 733-748 [PMID: 17668639 DOI: 10.1093/jnci/91.22.1933]
21 **Dignam JJ**, Colangelo L, Tian W, Jones J, Smith R, Wickerham DL, Wolmark N. Outcomes among African-Americans and Caucasians in colon cancer adjuvant therapy trials: findings from the National Surgical Adjuvant Breast and Bowel Project. *J Natl Cancer Inst* 1999; **91**: 1933-1940 [PMID: 10564677 DOI: 10.1093/jnci/91.22.1933]
22 **Doubeni CA**, Field TS, Buist DS, Korner EJ, Bigelow C, Lamerato L, Herrinton L, Quinn VP, Hart G, Hornbrook MC, Gurwitz JH, Wagner EH. Racial differences in tumor stage and survival for colorectal cancer in an insured population. *Cancer* 2007; **109**: 612-620 [PMID: 17186529 DOI: 10.1002/cncr.22437]
23 **Dominitz JA**, Samsa GP, Landsman P, Provenzale D. Race, treatment, and survival among colorectal carcinoma patients in an equal-access medical system. *Cancer* 1998; **82**: 2312-2320 [PMID: 9635522 DOI: 10.1002/(SICI)1097-0142(19980615)82]
24 **Fairley TL**, Cardinez CJ, Martin J, Alley L, Friedman C, Edwards B, Jamison P. Colorectal cancer in U.S. adults younger than 50 years of age, 1998-2001. *Cancer* 2006; **107**: 1153-1161 [PMID: 16862554 DOI: 10.1002/cncr.22012]
25 **Govindarajan R**, Shah RV, Erkman LG, Hutchins LF. Racial differences in the outcome of patients with colorectal carcinoma. *Cancer* 2003; **97**: 493-498 [PMID: 12518374]
26 **Le H**, Ziogas A, Lipkin SM, Zell JA. Effects of socioeconomic status and treatment disparities in colorectal cancer survival. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 1950-1962 [PMID: 18708384 DOI: 10.1158/1055-9965]
27 **Marcella S**, Miller JE. Racial differences in colorectal cancer mortality. The importance of stage and socioeconomic status. *J Clin Epidemiol* 2001; **54**: 359-366 [PMID: 11297886 DOI: 10.1016/S0895-4356(00)00316-4]
28 **Alexander D**, Jhala N, Chatla C, Steinhauer J, Funkhouser E, Coffey CS, Grizzle WE, Manne U. High-grade tumor differentiation is an indicator of poor prognosis in African Americans with colonic adenocarcinomas. *Cancer* 2005; **103**: 2163-2170 [PMID: 15816050 DOI: 10.1002/cncr.21021]
29 **Harris GJ**, Senagore AJ, Lavery IC, Church JM, Fazio VW. Factors affecting survival after palliative resection of colorectal carcinoma. *Colorectal Dis* 2002; **4**: 31-35 [PMID: 12780652 DOI: 10.1046/j.1463-1318.2002.00304.x]
30 **Samowitz WS**, Curtin K, Ma KN, Schaffer D, Coleman LW, Leppert M, Slattery ML. Microsatellite instability in sporadic colon cancer is associated with an improved prognosis at the population level. *Cancer Epidemiol Biomarkers Prev* 2001; **10**: 917-923 [PMID: 11535541]
31 **Alexander D**, Chatla C, Funkhouser E, Meleth S, Grizzle WE, Manne U. Postsurgical disparity in survival between African Americans and Caucasians with colonic adenocarcinoma. *Cancer* 2004; **101**: 66-76 [PMID: 15221990 DOI: 10.1002/cncr.20337]
32 **Edge SB**, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A. AJCC Cancer Staging Manual. 7th edition. New York: Springer, 2010: 649
33 **Compton CC**, Greene FL. The staging of colorectal cancer: 2004 and beyond. *CA Cancer J Clin* 2004; **54**: 295-308 [PMID: 15537574 DOI: 10.3322/canjclin.54.6.295]
34 **Cunningham D**, Atkin W, Lenz HJ, Lynch HT, Minsky B, Nordlinger B, Starling N. Colorectal cancer. *Lancet* 2010; **375**: 1030-1047 [PMID: 20304247 DOI: 10.1016/S0140-6736(10)60-353-4]
35 **Hyslop T**, Weinberg DS, Schulz S, Barkun A, Waldman SA. Occult tumor burden contributes to racial disparities in stage-specific colorectal cancer outcomes. *Cancer* 2012; **118**: 2532-2540 [PMID: 21887684 DOI: 10.1002/cncr.26516]
36 **Bach PB**, Schrag D, Brawley OW, Galaznik A, Yakren S, Begg CB. Survival of blacks and whites after a cancer diagnosis. *JAMA* 2002; **287**: 2106-2113 [PMID: 11966385 DOI: 10.1001/jama.287.16.2106]
37 **Advani AS**, Atkeson B, Brown CL, Peterson BL, Fish L, Johnson JL, Gockerman JP, Gautier M. Barriers to the participation of African-American patients with cancer in clinical trials: a pilot study. *Cancer* 2003; **97**: 1499-1506 [PMID: 12627515 DOI: 10.1002/cncr.11213]
38 **Gregg J**, Curry RH. Explanatory models for cancer among African-American women at two Atlanta neighborhood health centers: the implications for a cancer screening program. *Soc Sci Med* 1994; **39**: 519-526 [PMID: 7973851 DOI: 10.1016/0277-9536(94)90094-9]
39 **Schrag D**, Cramer LD, Bach PB, Begg CB. Age and adjuvant chemotherapy use after surgery for stage III colon cancer. *J Natl Cancer Inst* 2001; **93**: 850-857 [PMID: 11390534 DOI: 10.1093/jnci/93.11.850]
40 **Morris AM**, Billingsley KG, Hayanga AJ, Matthews B, Baldwin LM, Birkmeyer JD. Residual treatment disparities after oncology referral for rectal cancer. *J Natl Cancer Inst* 2008; **100**: 738-744 [PMID: 18477800 DOI: 10.1093/jnci/djn145]
41 **Super N**. Medigap: prevalence, premiums, and opportunities for reform. *NHPF Issue Brief* 2002; **(782)**: 1–23 [PMID: 12240698]
42 **Cooper GS**, Yuan Z, Landefeld CS, Rimm AA. Surgery for colorectal cancer: Race-related differences in rates and survival among Medicare beneficiaries. *Am J Public Health* 1996; **86**: 582-586 [PMID: 8604797 DOI: 10.2105/AJPH.86.4.582]
43 **Ball JK**, Elixhauser A. Treatment differences between blacks and whites with colorectal cancer. *Med Care* 1996; **34**: 970-984 [PMID: 8792784 DOI: 10.1097/00005650-199609000-00008]
44 **Demissie K**, Oluwole OO, Balasubramanian BA, Osinubi OO, August D, Rhoads GG. Racial differences in the treatment of colorectal cancer: a comparison of surgical and

radiation therapy between Whites and Blacks. *Ann Epidemiol* 2004; **14**: 215-221 [PMID: 15036226 DOI: 10.1016/j.annepidem.2003.08.002]

45 **Cook AD**, Single R, McCahill LE. Surgical resection of primary tumors in patients who present with stage IV colorectal cancer: an analysis of surveillance, epidemiology, and end results data, 1988 to 2000. *Ann Surg Oncol* 2005; **12**: 637-645 [PMID: 15965730 DOI: 10.1245/ASO.2005.06.012]

46 **Baldwin LM**, Dobie SA, Billingsley K, Cai Y, Wright GE, Dominitz JA, Barlow W, Warren JL, Taplin SH. Explaining black-white differences in receipt of recommended colon cancer treatment. *J Natl Cancer Inst* 2005; **97**: 1211-1220 [PMID: 16106026 DOI: 10.1093/jnci/dji241]

47 **McCollum AD**, Catalano PJ, Haller DG, Mayer RJ, Macdonald JS, Benson AB, Fuchs CS. Outcomes and toxicity in african-american and caucasian patients in a randomized adjuvant chemotherapy trial for colon cancer. *J Natl Cancer Inst* 2002; **94**: 1160-1167 [PMID: 12165641 DOI: 10.1093/jnci/94.15.1160]

48 **Sharma S**, O'Keefe SJ. Environmental influences on the high mortality from colorectal cancer in African Americans. *Postgrad Med J* 2007; **83**: 583-589 [PMID: 17823224 DOI: 10.1136/pgmj.2007.058958]

49 **Dobie SA**, Baldwin LM, Dominitz JA, Matthews B, Billingsley K, Barlow W. Completion of therapy by Medicare patients with stage III colon cancer. *J Natl Cancer Inst* 2006; **98**: 610-619 [PMID: 16670386 DOI: 10.1093/jnci/djj159]

50 **Yothers G**, Sargent DJ, Wolmark N, Goldberg RM, O'Connell MJ, Benedetti JK, Saltz LB, Dignam JJ, Blackstock AW; ACCENT Collaborative Group. Outcomes among black patients with stage II and III colon cancer receiving chemotherapy: an analysis of ACCENT adjuvant trials. *J Natl Cancer Inst* 2011; **103**: 1498-506 [DOI: 10.1093/jnci/djr310]

51 **Rabeneck L**, Souchek J, El-Serag HB. Survival of colorectal cancer patients hospitalized in the Veterans Affairs Health Care System. *Am J Gastroenterol* 2003; **98**: 1186-1192 [PMID: 12809847 DOI: 10.1111/j.1572-0241.2003.07448.x]

52 **Mandel JS**, Bond JH, Church TR, Snover DC, Bradley GM, Schuman LM, Ederer F. Reducing mortality from colorectal cancer by screening for fecal occult blood. Minnesota Colon Cancer Control Study. *N Engl J Med* 1993; **328**: 1365-1371 [PMID: 8474513 DOI: 10.1056/NEJM199305133281901]

53 **Schoen RE**, Pinsky PF, Weissfeld JL, Yokochi LA, Church T, Laiyemo AO, Bresalier R, Andriole GL, Buys SS, Crawford ED, Fouad MN, Isaacs C, Johnson CC, Reding DJ, O'Brien B, Carrick DM, Wright P, Riley TL, Purdue MP, Izmirlian G, Kramer BS, Miller AB, Gohagan JK, Prorok PC, Berg CD. Colorectal-cancer incidence and mortality with screening flexible sigmoidoscopy. *N Engl J Med* 2012; **366**: 2345-2357 [PMID: 22612596 DOI: 10.1056/NEJMoa1114635]

54 **Atkin WS**, Edwards R, Kralj-Hans I, Wooldrage K, Hart AR, Northover JM, Parkin DM, Wardle J, Duffy SW, Cuzick J. Once-only flexible sigmoidoscopy screening in prevention of colorectal cancer: a multicentre randomised controlled trial. *Lancet* 2010; **375**: 1624-1633 [PMID: 20430429 DOI: 10.1016/S0140-6736(10)60551-X]

55 **Levin B**, Lieberman DA, McFarland B, Andrews KS, Brooks D, Bond J, Dash C, Giardiello FM, Glick S, Johnson D, Johnson CD, Levin TR, Pickhardt PJ, Rex DK, Smith RA, Thorson A, Winawer SJ. Screening and surveillance for the early detection of colorectal cancer and adenomatous polyps, 2008: a joint guideline from the American Cancer Society, the US Multi-Society Task Force on Colorectal Cancer, and the American College of Radiology. *Gastroenterology* 2008; **134**: 1570-1595 [PMID: 18384785 DOI: 10.1053/j.gastro.2008.02.002]

56 **Fisher DA**, Dougherty K, Martin C, Galanko J, Provenzale D, Sandler RS. Race and colorectal cancer screening: a population-based study in North Carolina. *N C Med J* 2004; **65**: 12-15 [PMID: 15052704]

57 **Shapiro JA**, Seeff LC, Thompson TD, Nadel MR, Klabunde CN, Vernon SW. Colorectal cancer test use from the 2005 National Health Interview Survey. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 1623-1630 [PMID: 18628413 DOI: 10.1158/1055-9965.EPI-07-2838]

58 **Wilkins T**, Gillies RA, Harbuck S, Garren J, Looney SW, Schade RR. Racial disparities and barriers to colorectal cancer screening in rural areas. *J Am Board Fam Med* 2012; **25**: 308-317 [PMID: 22570394 DOI: 10.3122/jabfm.2012.03.100307]

59 **Benarroch-Gampel J**, Sheffield KM, Lin YL, Kuo YF, Goodwin JS, Riall TS. Colonoscopist and primary care physician supply and disparities in colorectal cancer screening. *Health Serv Res* 2012; **47**: 1137-1157 [PMID: 22150580 DOI: 10.1111/j.1475-6773.2011.01355]

60 **Doubeni CA**, Laiyemo AO, Klabunde CN, Young AC, Field TS, Fletcher RH. Racial and ethnic trends of colorectal cancer screening among Medicare enrollees. *Am J Prev Med* 2010; **38**: 184-191 [PMID: 20117575 DOI: 10.1016/j.amepre.2009.10.037]

61 **Tehranifar P**, Neugut AI, Phelan JC, Link BG, Liao Y, Desai M, Terry MB. Medical advances and racial/ethnic disparities in cancer survival. *Cancer Epidemiol Biomarkers Prev* 2009; **18**: 2701-2708 [PMID: 19789367]

62 **Lansdorp-Vogelaar I**, Kuntz KM, Knudsen AB, van Ballegooijen M, Zauber AG, Jemal A. Contribution of screening and survival differences to racial disparities in colorectal cancer rates. *Cancer Epidemiol Biomarkers Prev* 2012; **21**: 728-736 [PMID: 22514249 DOI: 10.1158/1055-9965.EPI-12-0023]

63 **Doubeni CA**, Laiyemo AO, Reed G, Field TS, Fletcher RH. Socioeconomic and racial patterns of colorectal cancer screening among Medicare enrollees in 2000 to 2005. *Cancer Epidemiol Biomarkers Prev* 2009; **18**: 2170-2175 [PMID: 19622721 DOI: 10.1158/1055-9965.EPI-09-0104]

64 **Doubeni CA**, Jambaulikar GD, Fouayzi H, Robinson SB, Gunter MJ, Field TS, Roblin DW, Fletcher RH. Neighborhood socioeconomic status and use of colonoscopy in an insured population--a retrospective cohort study. *PLoS One* 2012; **7**: e36392 [DOI: 10.1371/journal.pone.0036392]

65 **Fyffe DC**, Hudson SV, Fagan JK, Brown DR. Knowledge and barriers related to prostate and colorectal cancer prevention in underserved black men. *J Natl Med Assoc* 2008; **100**: 1161-1167 [PMID: 18942277]

66 **Carcaise-Edinboro P**, Bradley CJ. Influence of patient-provider communication on colorectal cancer screening. *Med Care* 2008; **46**: 738-745 [PMID: 18580394 DOI: 10.1097/MLR.0b013e318178935a]

67 **Seeff LC**, Nadel MR, Klabunde CN, Thompson T, Shapiro JA, Vernon SW, Coates RJ. Patterns and predictors of colorectal cancer test use in the adult U.S. population. *Cancer* 2004; **100**: 2093-2103 [PMID: 15139050]

68 **Merchant JL**, Omary MB. Underrepresentation of underrepresented minorities in academic medicine: the need to enhance the pipeline and the pipe. *Gastroenterology* 2010; **138**: 19-26.e1-19-26.e3 [PMID: 19944787 DOI: 10.1053/j.gastro.2009.11.017]

69 **Abreu MT**, Okolo PI. Re: underrepresentation of underrepresented minorities in academic medicine. *Gastroenterology* 2010; **139**: 359; author reply 359 [PMID: 20639091 DOI: 10.1053/j.gastro.2010.03.069]

70 **United States Preventive Services Task Force.** Screening for colorectal cancer: U.S. Preventive Services Task Force recommendation statement. *Ann Intern Med* 2008; **149**: 627-637 [PMID: 18838716]

71 **Gupta S**, Shah J, Balasubramanian BA. Strategies for reducing colorectal cancer among blacks. *Arch Intern Med* 2012; **172**: 182-184 [PMID: 22271128 DOI: 10.1001/archinternmed.2011.594]

72 **Qaseem A**, Denberg TD, Hopkins RH, Humphrey LL, Levine J, Sweet DE, Shekelle P. Screening for colorectal cancer: a guidance statement from the American College of Phy-

sicians. *Ann Intern Med* 2012; **156**: 378-386 [PMID: 22393133 DOI: 10.7326/0003-4819-156-5-201203060-00010]
73 **Rex DK**, Johnson DA, Anderson JC, Schoenfeld PS, Burke CA, Inadomi JM. American College of Gastroenterology guidelines for colorectal cancer screening 2009 [corrected]. *Am J Gastroenterol* 2009; **104**: 739-750 [PMID: 19240699 DOI: 10.1038/ajg.2009.104]
74 **Cash BD**, Banerjee S, Anderson MA, Ben-Menachem T, Decker GA, Fanelli RD, Fukami N, Ikenberry SO, Jain R, Jue TL, Khad KM, Krinsky ML, Malpas PM, Maple JT, Sharaf R, Dominitz JA. Ethnic issues in endoscopy. *Gastrointest Endosc* 2010; **71**: 1108-1112 [PMID: 20598241 DOI: 10.1016/j.gie.2010.03.001]
75 **Murff HJ**, Peterson NB, Fowke JH, Hargreaves M, Signorello LB, Dittus RS, Zheng W, Blot WJ. Colonoscopy screening in African Americans and Whites with affected first-degree relatives. *Arch Intern Med* 2008; **168**: 625-631 [PMID: 18362255 DOI: 10.1001/archinte.168.6.625]
76 **Inadomi JM**, Vijan S, Janz NK, Fagerlin A, Thomas JP, Lin YV, Muñoz R, Lau C, Somsouk M, El-Nachef N, Hayward RA. Adherence to colorectal cancer screening: a randomized clinical trial of competing strategies. *Arch Intern Med* 2012; **172**: 575-582 [PMID: 22493463 DOI: 10.1001/archinternmed.2012.332]
77 **Gupta S**, Halm EA, Rockey DC, Hammons M, Koch M, Carter E, Valdez L, Tong L, Ahn C, Kashner M, Argenbright K, Tiro J, Geng Z, Pruitt S, Skinner CS. Comparative effectiveness of fecal immunochemical test outreach, colonoscopy outreach, and usual care for boosting colorectal cancer screening among the underserved: a randomized clinical trial. *JAMA Intern Med* 2013; **173**: 1725-1732 [PMID: 23921906 DOI: 10.1001/jamainternmed.2013.9294]
78 **Laiyemo AO**, Doubeni C, Pinsky PF, Doria-Rose VP, Bresalier R, Lamerato LE, Crawford ED, Kvale P, Fouad M, Hickey T, Riley T, Weissfeld J, Schoen RE, Marcus PM, Prorok PC, Berg CD. Race and colorectal cancer disparities: health-care utilization vs different cancer susceptibilities. *J Natl Cancer Inst* 2010; **102**: 538-546 [PMID: 20357245 DOI: 10.1093/jnci/djq068]
79 **White A**, Vernon SW, Franzini L, Du XL. Racial and ethnic disparities in colorectal cancer screening persisted despite expansion of Medicare's screening reimbursement. *Cancer Epidemiol Biomarkers Prev* 2011; **20**: 811-817 [PMID: 21546366]
80 **Naylor K**, Ward J, Polite BN. Interventions to improve care related to colorectal cancer among racial and ethnic minorities: a systematic review. *J Gen Intern Med* 2012; **27**: 1033-1046 [PMID: 22798214 DOI: 10.1007/s11606-012-2044-2]
81 **Richards CA**, Kerker BD, Thorpe L, Olson C, Krauskopf MS, Silver LS, Weber TK, Winawer SJ. Increased screening colonoscopy rates and reduced racial disparities in the New York Citywide campaign: an urban model. *Am J Gastroenterol* 2011; **106**: 1880-1886 [PMID: 22056567 DOI: 10.1038/ajg.2011.191]
82 **Grubbs SS**, Polite BN, Carney J, Bowser W, Rogers J, Katurakes N, Hess P, Paskett ED. Eliminating racial disparities in colorectal cancer in the real world: it took a village. *J Clin Oncol* 2013; **31**: 1928-1930 [PMID: 23589553 DOI: 10.1200/JCO.2012.47.8412]

P- Reviewers: Bloomston M, NaitoY **S- Editor**: Qi Y
L- Editor: A **E- Editor**: Wu HL

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.877

World J Gastroenterol 2014 January 28; 20(4): 877-887
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Evolving treatment strategies for colorectal cancer: A critical review of current therapeutic options

Daniel C Damin, Anderson R Lazzaron

Daniel C Damin, Division of Coloproctology, Department of Surgery, Hospital de Clinicas de Porto Alegre, Federal University of Rio Grande do Sul, Porto Alegre, RS 90035-903, Brazil
Anderson R Lazzaron, Postgraduate Program in Surgery, Federal University of Rio Grande do Sul, Porto Alegre, RS 90035-903, Brazil
Author contributions: Damin DC and Lazzaron AR contributed equally to this work by reviewing literature and writing the paper.
Correspondence to: Daniel C Damin, MD, PhD, Division of Coloproctology, Department of Surgery, Hospital de Clinicas de Porto Alegre, Federal University of Rio Grande do Sul, Rua Ramiro Barcelos 2350, Sala 600, Porto Alegre, RS 90035-903, Brazil. damin@terra.com.br
Telephone: +55-51-96020442 Fax: +55-51-33285168
Received: September 27, 2013 Revised: November 22, 2013
Accepted: January 6, 2014
Published online: January 28, 2014

Abstract

Management of rectal cancer has markedly evolved over the last two decades. New technologies of staging have allowed a more precise definition of tumor extension. Refinements in surgical concepts and techniques have resulted in higher rates of sphincter preservation and better functional outcome for patients with this malignancy. Although, preoperative chemoradiotherapy followed by total mesorectal excision has become the standard of care for locally advanced tumors, many controversial matters in management of rectal cancer still need to be defined. These include the feasibility of a non-surgical approach after a favorable response to neoadjuvant therapy, the ideal margins of surgical resection for sphincter preservation and the adequacy of minimally invasive techniques of tumor resection. In this article, after an extensive search in PubMed and Embase databases, we critically review the current strategies and the most debatable matters in treatment of rectal cancer.

Key words: Rectal cancer; Colorectal cancer; Staging; Sphincter preservation; Neoadjuvant chemo-radiotherapy; Surgery

Core tip: Rectal cancer management is currently a multidisciplinary effort, which incorporates new concepts and technologies, resulting in significant improvement in patients' oncological and functional outcomes. Despite the evolution reported in the last decades, there are still many unanswered questions about treatment of rectal cancer. In this article, we critically analyzed the main controversial matters in current rectal cancer management.

Damin DC, Lazzaron AR. Evolving treatment strategies for colorectal cancer: A critical review of current therapeutic options. *World J Gastroenterol* 2014; 20(4): 877-887 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/877.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.877

INTRODUCTION

In the last two decades there have been significant changes in evaluation and management of rectal cancer. Incorporation of new technologies of staging and new therapeutic concepts has improved oncologic and functional results in patients with this malignancy. Treatment of the disease has become a multidisciplinary effort, which depends upon the integration between oncologists and colorectal surgeons. The goal of this article is to analyze the main controversial matters in current rectal cancer management.

LITERATURE SEARCH

We performed a literature search using PubMed and

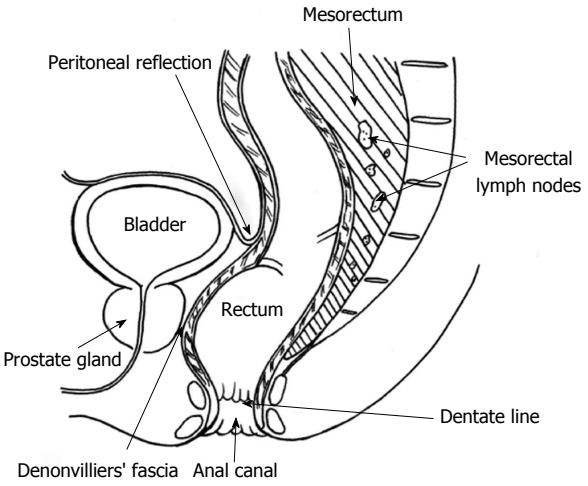


Figure 1 Rectal anatomy.

Embase databases up to September 2013. The following MeSH search terms were used: rectal cancer, tumor staging, total mesorectal excision, radiotherapy, chemoradiotherapy, neoadjuvant chemo-radiotherapy, "watch and wait", surgery and sphincter preservation. These terms were applied in various combinations to maximize the search. Only articles written in English were included. Additional searches of the embedded references from primary articles were performed to further improve the review.

ANATOMIC DEFINITIONS

Some anatomic concepts are essential for planning rectal cancer therapy (Figure 1). The rectum is the segment of the large bowel located between the sigmoid colon and the anal canal. Its upper limit is generally located at the level of the sacral promontory, roughly corresponding to a point where the taeniae coli spread out and can no longer be distinguished. For practical purposes, it is accepted that malignant tumors located within 15 cm from the anal verge (using a rigid proctoscope) should be diagnosed as rectal cancers. Below the peritoneal reflection, the rectum has no serosal layer and is surrounded by a circumferential fatty sheath known as mesorectum. It contains the perirectal lymph nodes, which usually represent the first sites to which rectal tumors disseminate[1,2].

PRETREATMENT EVALUATION AND STAGING

The process of staging a rectal cancer starts with a careful physical examination. Digital examination of the rectum, along with proctoscopy, should determine degree of tumor fixation, percentage of the circumference involved, distance of the tumor form the anal verge and likelihood of sphincter preservation. Vaginal exam may reveal direct tumoral invasion. Inguinal lymph nodes should be examined if tumor arises from the lower third of the rectum. According to the the American Society of Colon and Rectal Surgeons 2013 guidelines[2], for preoperative staging a complete colonoscopy should be performed, if the tumor is not obstructive, for histologic confirmation of the diagnosis and to rule out proximal synchronous lesions. In cases in which a full colonoscopy cannot be performed, a preoperative double-contrast barium enema or a computed tomography (CT) colonography may be used. Alternatively, for patients with incomplete preoperative colonoscopy, intraoperative colonoscopy may be used as an effective method to detect synchronous lesions[3].

Table 1 Tumor node metastasis clinical classification (colon and rectum cancer)

TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma *in situ*: intraepithelial or invasion of lamina propria
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades subserosa or into non-peritonealized pericolic or perirectal tissues
T4	Tumor directly invades other organs or structures and/or perforates visceral peritoneum
T4a	Tumor perforates visceral peritoneum
T4b	Tumor directly invades other organs or structures
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1-3 regional lymph nodes
N1a	Metastasis in 1 regional lymph node
N1b	Metastasis in 2-3 regional lymph nodes
N1c	Tumor deposit(s), *i.e.*, satellites, in the subserosa, or in non-peritonealized pericolic or perirectal soft tissue without regional lymph node metastasis
N2	Metastasis in 4 or more regional lymph nodes
N2a	Metastasis in 4-6 regional lymph nodes
N2b	Metastasis in 7 or more regional lymph nodes
M0	No distant metastasis
M1	Distant metastasis
M1a	Metastasis confined to one organ [liver, lung, ovary, non-regional lymph node(s)]
M1b	Metastasis in more than one organ or the peritoneum

AJCC: American joint committee on cancer[4].

In order to complete pretreatment staging, a CT scan of the thorax and abdomen and pelvis, and the measurement of serum carcinoembryonic antigen are recommended[2]. Once distant metastases have been ruled out, the most important factor to define the strategy of treatment to be adopted is the locoregional staging. Tumor node metastasis (TNM) system, as recommended by the American Joint Committee on Cancer, is currently the most widely used system for staging rectal adenocarcinomas (Tables 1 and 2)[4].

There is considerable controversy on what is the ideal method to evaluate local extent of the tumor. Although CT scan is no longer considered the modality of choice, it can be used in patients with primarily advanced T-stage tumors (accuracy of 79% to 94%). Accuracy fells to 52% to 74% when smaller and less advanced tumors are analyzed[5-9]. Endorectal ultrasound (EUS) and magnetic resonance imaging (MRI) with either endorectal or increasingly phase array coils are currently the modalities

Table 2 Tumor node metastasis stage grouping (colon and rectum cancer)

Stage 0	Tis	N0	M0
Stage Ⅰ	T1, T2	N0	M0
Stage Ⅱ	T3, T4	N0	M0
Stage ⅡA	T3	N0	M0
Stage ⅡB	T4a	N0	M0
Stage ⅡC	T4b	N0	M0
Stage Ⅲ	Any T	N1, N2	M0
Stage ⅢA	T1, T2	N1	M0
	T1	N2a	M0
Stage ⅢB	T3, T4a	N1	M0
	T2, T3	N2a	M0
	T1, T2	N2b	M0
Stage ⅢC	T4a	N2a	M0
	T3, T4a	N2b	M0
	T4b	N1, N2	M0
Stage ⅣA	Any T	any N	M1a
Stage ⅣB	Any T	any N	M1b

AJCC: American joint committee on cancer[4].

of choice for the local staging. EUS is considered more precise (T-stage accuracy ranging from 75% to 95%), as compared to MRI (T-stage accuracy ranging from 59% to 95%). However, the efficacy of EUS is limited in stenotic or large bulky lesions which cannot be traversed by the probe[10-12].

Evaluation of perirectal lymph nodes is still a major controversial matter, particularly because metastases can be found in normal-sized lymph nodes[13,14]. According to a meta-analysis of 90 studies[15], none of the three imaging modalities were significantly superior in defining lymph node involvement. Sensitivities and specificities of the exams were as follows: CT (55% to 74%), EUS (67% to 78%), and MRI (66% to 76%).

TREATMENT CONTROVERSIES

Local excision

Despite limitations of the imaging exams, they are essential to define the strategy of treatment to be adopted. Tumors classified as T1 with no evidence of nodal involvement may be amenable to local excision, depending upon some specific criteria. These include: well to moderately differentiated carcinomas, measuring less than 3 cm in diameter, occupying less than a third of the circumference of the rectal lumen, with no lymphatic, vascular or perineural invasion[16,17]. Using these selection criteria, it can be achieved 10-year overall survival rates of 84% and disease-free survival of 75%[18].

There are currently two main techniques for local excision. The first one is transanal resection which consists of excision of all layers of the bowel wall, including perirectal fat, with disease-free margins of at least one centimeter. It is a procedure that colorectal surgeons are familiar with, however its indication is limited to tumors located within 8 cm from the anal verge[19]. The second method is transanal endoscopic microsurgery (TEM), which utilizes a special proctoscope with a 3D binocular optic and a set of endoscopic surgical instruments that permit resection of tumors located up to 20 cm from the anal verge. To this moment, there are few studies comparing TEM with transanal resection or with radical surgery[20,21]. However, the published trials have suggested the technique is relatively safe, usually with minor complications.

It is important to take into account that even in well-selected patients (cT1N0), the risk of lymph nodes metastases can reach 10%. The most challenging aspect in selecting patients for that sort of treatment is that the preoperative staging remains limited in defining precisely tumor invasion and nodal involvement. Considering that the local recurrence rate may vary from 26% to 47% for T2 lesions, a radical resection must be indicated for patients with these lesions. However, if the patient is a poor surgical candidate, we could alternatively recommend complementary chemoradiation to reduce the risk of local recurrence[18].

NEOADJUVANT TREATMENT

According to the current evidence, tumors classified as TNM stage Ⅱ or Ⅲ (T3/T4, N1/N2) should receive neoadjuvant treatment before radical resection[22]. Two main options of preoperative therapy have been proposed. The first is short-course radiotherapy, which is considered the treatment of choice in North-European countries and Scandinavia. The second option is the so-called long-course preoperative chemoradiotherapy, which is the favored treatment in most European countries and in the United States. Characteristics and results of the main trials investigating the neoadjuvant strategies in rectal carcinoma are presented in Table 3.

Recently, the German Rectal Cancer Study Group published results of their trial after a median follow-up of 11 years[31]. Overall survival at 10 years was 59.6% in the preoperative arm and 59.9% in the postoperative arm (P = 0.85). The 10-year cumulative incidence of local relapse was 7.1% and 10.1% in the preoperative and postoperative groups, respectively (P = 0.048), representing a small absolute reduction of long-term local recurrence (3%). No significant differences were detected for 10-year incidence of distant metastases (29.8% and 29.6%) and disease-free survival.

A recently published meta-analysis[32] assessed effectiveness and safety of neoadjuvant radiotherapy in rectal cancer. The authors searched several database, analyzing randomized controlled trials comparing either neoadjuvant therapy *vs* surgery alone (17 trials including 8568 patients) or neoadjuvant chemoradiotherapy *vs* neoadjuvant radiotherapy (5 trials including 2.393 patients). Neoadjuvant radiotherapy decrease local recurrence (HR = 0.59; 95%CI: 0.48-0.72) compared to surgery alone even after total mesorectal excision. It has marginal benefit in overall survival (HR = 0.93; 95%CI: 0.85-1.00), but was associated with increased perioperative mortality (HR = 1.48; 95%CI: 1.08-2.03). Neoadjuvant chemoradiation improved local control as compared to radiotherapy alone (HR = 0.53; 95%CI: 0.39-0.72), but both treatments had

Table 3 Major neoadjuvant therapy trials

Ref.	*n*	Treatment arms	Local recurrence rate	Overall survival rate
Upsala Trial[23]	471	Arm 1 (236): preoperative RT (25.5 Gy delivered in 5-7 d) Arm 2 (235): postoperative RT (60 Gy delivered in 8 wk)	5 yr of follow-up Arm 1: 12% Arm 2: 21% *P* = 0.02	5-yr survival rate Arm 1: 42% Arm 2: 38% *P* = 0.42
Stockholm I Trial[24]	849	Arm 1 (423 patients): 25 Gy during 5-7 d followed by surgery Arm 2 (421 patients): surgery alone	Median follow-up time of 107 mo Arm 1: 14% Arm 2: 28% *P* < 0.01	Median follow-up time of 107 mo No significant difference between groups
Swedish Rectal Cancer Trial[25]	1168	Arm 1 (553 patients): preoperative RT - 25 Gy delivered in five fractions in 1 wk, followed by surgery Arm 2 (557 patients): Surgery alone	5 yr of follow-up Arm 1: 11% Arm 2: 27% *P* < 0.001	5-yr survival rate Arm 1: 58.0% Arm 2: 48.0% *P* = 0.004
Dutch TME Trial[26]	1861	Arm 1 (924 patients): preoperative RT (5 Gy × 5 d) followed by TME Arm 2 (937): TME alone	2 yr of follow-up Arm 1: 2.4% Arm 2: 8.2% *P* < 0.001	2-yr survival rate Arm 1: 82.0% Arm 2: 81.8% *P* = 0.84
Stockholm II Trial[27]	557	Arm 1 (272): preoperative radiotherapy (25 Gy in one week) followed by surgery within a week Arm 2 (285): surgery alone	Median follow-up was 8.8 yr Arm 1: 12% Arm 2: 25% *P* < 0.001	Median follow-up 8.8 yr Arm 1: 39% Arm 2: 36% *P* = 0.2
German Rectal Cancer Study Group[28]	823	Arm 1 (421 patients): preoperative CHRT: 50.4 Gy/28 fractions/5 fractions weekly and fluorouracil (continuous infusion) in first and fifth week of RT. TME after 6 wk Additional 4 cycles of FU every 4 wk Arm 2 (402 patients): postoperative CHRT (same as in Arm 1 except a 5.4 Gy boost in RT)	5 yr of follow-up Arm 1: 6.0% Arm 2: 13% *P* = 0.006	5-yr survival rate Arm 1: 76.0% Arm 2: 74.0% *P* = 0.80
Polish Rectal Cancer Trial[29]	312	Arm 1 (155 patients): preoperative RT (5 Gy × 5 d) followed by TME at 7 d after RT Arm 2 (157 patients): preoperative RT (45 Gy/25 fractions/5 wk) + 2 cycles of chemotherapy on weeks 1 and 5 of RT followed by TME 4-6 wk later. The cycle consisted of leucovorin + fluorouracil both administered as rapid infusion on 5 consecutive days	4 yr of follow-up Arm 1: 9% Arm 2: 14.2% *P* = 0.170	4-yr survival rate Arm 1: 67.2% Arm 2: 66.2% *P* = 0.960
MRC CR07 and NCIC CTG C016[30]	1350	Arm 1 (674 patients): short-course radiotherapy (25 Gy/5 fractions) followed by surgery. Arm 2 (676 patients): initial surgery with selective post-operative chemoradiotherapy (45 Gy in 25 fractions plus 5-fluorouracil) restricted to patients with involvement of the circumferential resection margin.	3 yr of follow-up Arm 1: 4.0% Arm 2: 10.6% *P* < 0. 01	Estimated 5-yr survival rate Arm 1: 70.3% Arm 2: 67.9% *P* = 0.40

RT: Radiotherapy; TME: Total mesorectal excision; CHRT: Chemoradiotherapy; FU: Fluorouracil.

no influence in long-term survival.

One of the potential advantages of the neoadjuvant treatments is the possibility of tumor shrinkage, which, in theory, could increase the chance of performing a sphincter saving surgery. This hypothesis has been investigated in several trials using different regimens of preoperative treatment[30,33]. Recently, Gerard *et al*[34] published the results of a well-conducted systematic review on the impact of the neoadjuvant treatments in sphincter preservation. Seventeen randomized trials published between 1988 and 2009 were analyzed. The rate of sphincter saving surgery increased from 30% for the patients operated in the 80's[18] up to 77% in 2008[15]. However, in none of the main trials analyzed (except three trials with low number of patients) it was possible to demonstrate a significant benefit of the neoadjuvant treatment on the rate of sphincter preserving surgery. According to the authors, this increase in sphincter preservation appears to be the result of new technologies and changes in surgical concepts, such as incorporation of total mesorectal excision (TME) and the techniques for very low anastomosis.

These findings are in line with a previous study by Bujko *et al*[35] in which 10 randomized trials were reviewed. These studies included 4596 patients in whom preoperative chemoradiotherapy resulted in tumor shrinkage in the neoadjuvant arm as compared with the control arm. As acknowledged by the authors, there were several difficulties in comparing the studies, including different preoperative radiochemotherapy schemes, duration of the interval between radiotherapy and surgery and patient populations. Despite these limitations, it was concluded that preoperative radiotherapy does not have a positive impact on the rate of anterior resection and sphincter preservation.

Table 4 Locoregional recurrence in patients with complete clinical response who did not proceed to rectal resection *n* (%)

Ref.	No. of patients	T2	Radiotherapy	Chemotherapy	Complete clinical response	Locoregional recurrence
Nakagawa *et al*[41]	52	No	45-50.4 Gy, 28 fractions, 38 d	Fluorouracil + leucovorin	10 (19.2)	8 (80)
Habr-Gama *et al*[42]	360	Yes (14%)	50.4 Gy, 28 fractions, 5-6 wk	Fluorouracil + leucovorin	99 (27.5)	6 (6)
Lim *et al*[43]	48	T1/t2 (33%)	Mean 50 Gy, 25 fractions	Fluorouracil	27 (56)	11 (23)
Hughes *et al*[44]	58	No	45 Gy, 25 fractions, 33 d	Fluorouracil + leucovorin	10 (17)	6 (60)
Dalton *et al*[45]	49	No	45 Gy, 25 fractions, 33 d	Capecitabine	12 (24)	6 (50)
Maas *et al*[46]	192	Yes (24%)	50.4 Gy, 28 fractions, 6 wk	Capecitabine	21 (10.9)	1 (5)

"WAIT AND SEE" APPROACH

In about 10%-20% of patients with rectal cancer who receive preoperative chemoradiation, a pathological complete response (pCR), characterized by absence of viable tumor cells within the surgical specimen, can be expected[36]. According to a systematic review and meta-analysis of the literature, patients with pCR have better oncologic outcomes as compared with those presenting a less marked response to chemoradiation, including better rates of local recurrence, distant metastases, disease-free and overall survival at 5 years[37].

According to some authors, it is therefore valid to think that patients whose tumors have been sterilized by chemoradiation would have no additional benefit from a subsequent radical resection. In 1998, Habr-Gama *et al*[38] from Brazil proposed the "wait and see" policy for patients who achieve what they called complete clinical response (cCR) after neoadjuvant treatment. They evaluated 118 patients treated by preoperative CRT (50.4 Gy and concurrent 5-FU and leucovorin for 3 consecutive days on the first and last 3 d of radiotherapy). All patients underwent repeat evaluation and biopsy of any suspected residual lesions or scar tissue. Thirty-six patients (30.5%) were classified as being complete responders. In only six of these patients, complete response was confirmed by the absence of tumor in the surgical specimen. In the other 30 patients, a complete response was assumed by the absence of symptoms and negative findings on physical examination, biopsy and imaging tests during a median follow-up of 36 mo. Out of the later group, eight patients presented local failure, demanding salvage resection. The outcome for patients without recurrence was similar to that of patients found at surgery to have achieved a pCR. The authors concluded that about 26% of their patients could be spared from surgical resection using that conservative strategy of management.

In subsequent publications Habr-Gama *et al*[39] repeatedly reported favorable results. In 2006, they reported the outcome of 361 patients with distal rectal cancer managed by neoadjuvant chemoradiation. One hundred twenty-two patients were considered to have complete clinical response and were not immediately operated on. Of them, 99 patients sustained complete clinical response for at least 12 mo and were considered stage c0 (27.4%). There were 13 recurrences, but only six of these cases had local recurrent disease. Overall and disease-free 5-year survivals were 93% and 85% respectively.

Such good results, however, could not be reproduced by other groups. Nyasavajjala *et al*[40] reviewed pathologic results of patients operated on for rectal cancer after long course neoadjuvant chemoradiotherapy in two different tertiary British hospitals. One hundred and thirty-two consecutive patients were treated between 2002 and 2007. Only 13 out of 132 (10%) of patients had a complete pathological response, representing one-third of the cCR previously reported. They concluded that nonsurgical therapy for rectal cancer according to Habr-Gama algorithm of treatment may only be effective in a very small proportion of patients and could not be recommended. As shown in Table 4, there is a wide variation among studies in the rates of local recurrence for patients with cCR to chemoradiation who were not submitted to a subsequent rectal resection.

Recently, Glynne-Jones *et al*[36] conducted a systematic review of studies evaluating non-operative treatments and the "wait and see" strategy in rectal cancer. Most studies were retrospective and there were no randomized phase Ⅱ or phase Ⅲ trials. In all they could evaluate nine series, including 650 patients: 361 patients from the Habr-Gama series and 289 patients from the eight remaining series. The results in terms of cCR and local recurrence reported in the Brazilian series were clearly superior. However, there were significant heterogeneity among studies in terms of treatment regimen, methods of assessment used to define a cCR (digital exam, biopsy or imaging tests) and the follow-up strategy used. The authors concluded that evidence for the "wait and see" policy comes mainly from a single retrospective series, which included highly selected cases. Results obtained in patients with small rectal cancers cannot be extrapolated at this moment to patients with more advanced tumors where nodal involvement can be anticipated.

The main obstacle to implement the "wait-and-see" policy is the current lack of accuracy of the tests in determining whether there has really been a complete pathological response[36]. Due to changes in pelvic tissues after radiotherapy (edema, inflammation, fibrosis) it is very difficult to assess whether an apparent clinical response will eventually translate into a complete pathological response. Digital exam, proctoscopy and imaging tests (endoanal ultrasound, MRI or positron emission tomography-CT) are still imprecise for detecting microscopic tumor deposits within the rectum and perirectal lymph nodes[47]. Thus, the non-surgical approach remains experimental at this moment. Prospective multi-

institutional controlled studies based on uniform inclusion criteria are needed to define the efficacy and risks of this form of treatment.

RADICAL SURGICAL APPROACH

Surgery remains as the cornerstone curative treatment for rectal cancer. Sphincter preservation must be seen as a secondary objective, which should not compromise oncological adequacy of resection. Radical surgical treatment for rectal cancer, which consists of the resection of the rectum and lymphadenectomy, includes two main procedures: low anterior resection (LAR) and abdominoperineal resection (APR). In LAR, the anal sphincter complex is preserved and it is possible to restore intestinal continuity. In APR, the anal sphincters are resected *en bloc* and it is necessary to construct a definitive colostomy. Currently, LAR is the most commonly used surgery for the rectal cancer, while APR is applied in cases where it is not possible to get free margins without resecting the anal sphincter complex[48].

As a rule, the lower is the level of a rectal cancer, the worse is its prognosis. In a series[49] in which 2136 patients underwent radical surgeries with TME, the local recurrence rate was 15%, 13% and 9% for tumors located in inferior, medium and superior rectum, respectively. The correspondent five-year survival rates were 59%, 62% and 69%. The rate of local recurrence was 10% in patients submitted to LAR compared with 15% for patients submitted to APR. In addition, the five-year survival rate was 68% for LAR and 55% for APR. A similar study[50] analyze patients who underwent radical surgeries without any adjuvant therapy. The mean rate of local recurrence rate was 18.5% (19.3% for APR and 16.2% for LAR). There is currently strong evidence in literature showing that LAR and APR have similar long-term oncological results when appropriate surgical margins can be assured.

A diverting ostomy is strongly recommended for patients undergoing a LAR, particularly when a low anastomosis is constructed. A meta-analysis[51] of 4 randomized controlled trials and 21 non-randomized studies, including 11429 patients, showed more favorable results in patients with a diverting ostomy as compared with those without a protective stoma. Meta-analysis of the randomized trials showed lower rates of clinical anastomotic leak (RR = 0.39; $P < 0.001$) and reoperation (RR = 0.29; $P < 0.001$) in the stoma group. Similarly, meta-analysis of the non-randomized studies demonstrated lower rates of clinical anastomotic leak (RR = 0.74; $P < 0.001$), reoperation (RR = 0.28; $P < 0.001$) and mortality (RR = 0.42; $P < 0.001$) in the stoma group. A protective ostomy may be either a colostomy (usually in the transverse colon) or an ileostomy. The latter is more frequently used because it is technically easier to reverse and less associated with stoma prolapse. Usually, the ileostomy reversal is undertaken within 8 to 12 wk after the primary rectal resection[52].

DISTAL MARGIN OF RESECTION

At present, a distal mural margin of 2cm is considered the standard for rectal cancer resections[2]. One cm distal margin is accepted for low rectal tumors, if it is necessary to avoid an APR[2,53-56], because distal intramural spread occurs over 1 cm in only 4%-10% of the cases[57,58]. Ueno *et al*[59] demonstrated in a retrospective analysis of 80 patients who underwent APR that intra-mural distal spread occurs in 10.6% of the patients. In only 2.3% of the cases tumor cells can be found more than 1cm from the tumor, mainly in poor differentiated carcinomas. There are some authors that accept margins even shorter than the 1 cm[60]. In a recent meta-analysis evaluating 17 studies including 7097 patients the local recurrence rate was only 1% higher in the group of patients with distal margins less than 1 cm when compared to the group with distal margins measuring more than 1 cm (95%CI: 0.6-2.7, $P = 0.175$). They were not able to find a statistically significant difference in either local control or survival with margins of less than 1 cm. Analysis of a subgroup of patients with negative margins as close as 5 mm to the lower tumor border suggests it can be safely adopted in histologically favorable tumors[60]. To this date, however, there is no study establishing valid criteria for selecting patients to resection with less than 1 cm distal margin.

TOTAL MESORECTAL EXCISION

TME was proposed and made popular by Heald *et al*[61] in 1982, being recommended for tumors of the mid and lower rectum. It consists of complete excision of all the mesorectal tissue evolved by the visceral layer of endopelvic fascia, which must be kept intact and the circumferential margins not compromised. TME is based on the observation that viable tumor cells can be found within the mesorectum as far as 3 to 4 cm from the tumor lower border[62,63]. The technique consists of sharp dissection without the use of blunt instruments (including fingers) on the natural avascular plane between visceral and the parietal endopelvic fascia layers. That dissection requires the removal of the Denonvilliers fascia, especially when the tumor is anterior. The hypogastric and parasympathetic pelvic nerves must be preserved, avoiding urinary and sexual disfunction. Circumferential margins should be widely resected to reduce rates of local recurrence[64,65].

Since the introduction of TME, the five-year survival rates increased from 45%-50% to 75% and the local recurrence rates decreased from 30% to 5%-8%[66]. It is not necessary, however, to perform a TME in upper rectal tumors. Resecting a 5 cm distal margin of the mesorectum below the inferior tumor edge (partial mesorectal excision) is enough in those cases[62].

SPHINCTER PRESERVATION IN ULTRA-LOW RECTAL TUMORS

Sphincter preservation remains a challenge in low rectal

tumors. Whenever safe distal margins cannot be achieved, an APR is still the treatment of choice. However, for tumors located within 6 cm from the anal verge some conservative surgical procedures may be attempted[67,68]. In the ultra-LAR (uLAR), the rectal transection is performed transanally, with straight view to the inferior tumor board, and a manual coloanal anastomosis (CAA). Another alternative is the intersphincteric resection (ISR), in which the internal anal sphincter is partially or totally resected in order to obtain appropriate longitudinal and radial margins[68].

Recently, Rullier *et al*[68] have tried to standardize surgical treatment for the inferior rectal tumors, proposing a new classification of these tumors according its location and degree of sphincter invasion. They subdivided low rectal tumors into four categories: Type Ⅰ (supra-anal tumor): inferior tumor board located more than 1 cm from the anal ring; Type Ⅱ (juxta-anal tumor): inferior tumor board is located ≤ 1 cm distant from the anal ring; Type Ⅲ (intra-anal tumor): there is internal sphincter invasion; and Type Ⅳ (transanal tumor): when there is external sphincter or levator ani muscle invasion. In this study, Type Ⅰ cancers were treated through a conventional CAA, that was a Park procedure, including anal mucosectomy above the dentate line and preservation of the anal internal sphincter. Type Ⅱ tumors underwent partial ISR to achieve sphincter-preserving surgery with 1 cm distal resection margin. Type Ⅲ lesions had a total ISR removing the whole of the internal sphincter. Type Ⅳ lesions were treated through APR. Using that classification, the authors operated 404 cases, with local recurrence rates of 6%, 5%, 9% and 17% respectively for the types Ⅰ, Ⅱ, Ⅲ and Ⅳ (P = 0.186). Only 50% of the patients had good fecal continence while 11% had a severe fecal incontinence. Besides, 6% of their patients needed definitive colostomy due to postoperative fecal incontinence. As the authors recognized, results of their retrospective series need to be confirmed in prospective clinical trials.

Whenever a very low colorectal anastomosis is performed, the rectal reservoir is lost and it can result in the so-called anterior resection syndrome (soiling, urgency, multiple defecation). In this context, it is interesting to observe that some studies demonstrated that the quality of life of patients who have undergone uLAR may be inferior when compared to the one of patients submitted to APR[69]. Functional results of ISR are recognizably suboptimum[68,70], with only 50% of patients maintaining fecal continence after two years[68,71]. Thus, ISR must be considered for patients with adequate sphincteric function, as demonstrated by manometric evaluation of anal sphincters, and for those that can accept that functional results may be suboptimum.

SURGICAL SPECIALIZATION

The specialization degree of the surgeon is an important prognostic factor for the treatment of colorectal cancer. Patients operated by surgeons specialized in colorectal surgery have better outcome[72]. Regarding the rectal cancer, some studies confirm the influence of surgical specialization on the prognosis. In a population based audit[73] with 8219 cases, patients who have undergone elective proctectomy by colorectal surgeons obtained higher sphincter preservation rates as compared to the ones operated by general surgeons (OR = 1.42; P = 0.018). In a historical cohort study[74] involving five general hospitals with 683 patients operated on with curative intent, cancer-free five-year survival rate was significantly higher in the group operated by colorectal surgeons (HR = 1.5; P = 0.03). Similarly, a recent review and meta-analysis[75] demonstrated that patients operated by colorectal surgeons presented a lower rate of permanent ostomy (RR = 0.7; 95%CI: 0.53-0.94). In addition, in a retrospective study[76] with 384 consecutive rectal cancer patients, the results were significantly better when patients were operated by colorectal surgeons. After multivariate analysis, the five-year survival was 77% for patients operated by specialists and 68% for the patients operated by general surgeons. There was also a better local control as well as a higher rate of sphincter preservation in patients operated by specialists.

MINIMALLY INVASIVE SURGERY

Current evidence indicates that laparoscopic TME has equivalent oncological results as compared to open TME, when performed by experienced laparoscopic surgeons[2]. Most studies reported similar oncological results when comparing both techniques[77-81]. In a meta-analysis[82] involving 17 trials with 3158 patients with rectal cancer submitted to curative operations there was a statistically significant difference in the average number of recovered lymph nodes (laparoscopy = 10, open = 12, P = 0.001). However, it had no impact on the clinical outcome of the patients. Furthermore, there was no difference in the radial, proximal or distal margin status between the groups. In the CLASICC Trial[83], a single-institution clinical trial with 253 patients, the incidence of positive radial margins was 12% in the laparoscopic LAR group *vs* 6% for the open LAR one. That difference, however, was not statistically significant and there was no difference regarding local recurrence within five years.

Four prospective trials including 886 patients have reported no significant difference in disease-free or overall survival between the laparoscopic and open groups with a follow-up ranging from 37 to 113 mo[77-80]. In the COLOR Ⅱ trial[84], a multicenter randomized clinical trial with 1103 patients, there was no significant difference between the open surgery group and the laparoscopy group in relation to radial and distal margin status and in the number of recovered lymph nodes. A definitive answer regarding the safety and effectiveness of the rectal cancer laparoscopic surgery, however, still depends on the results of multicenter studies such as ACOSOG-Z6051 trial[85], currently conducted in the United States.

The recent introduction of the robotic surgical system has revolutionized the field of minimally invasive surgery. It provides several technical advances in relation

to laparoscopic surgery: better ergonomics, stable camera control, high-definition three-dimensional vision, filter of physiologic tremor and human wrist-like motion of robotic instruments[86]. Robotic technology also eliminates the fatigue associated with conventional laparoscopy and offers more comfort to the surgeon[87]. Another advantage of robotic surgery is a shorter learning curve when compared to laparoscopy, although cost of robotic surgery is significantly higher[88].

Several technical issues of the robotic surgery, however, should be carefully taken into account. There is a loss of tactile sensation with the robotic approach, which results in lack of tensile feedback to the surgeon. It can cause excessive traction of tissues and damage to anatomic structures, particularly during the initial experiences with the technique. Operative time is usually longer using the robotic system as compared with the laparoscopic approach, particularly because docking and separation of the robotic instruments from patient is a time consuming procedure. The patient's surgical position cannot be modified without undocking the robotic instruments, which may result in prolonged operative time and potential delay in conversion to open surgery if it is eventually necessary[88,89].

Recent studies have compared robotic surgery to laparoscopy for the treatment of rectal cancer. A meta-analysis[89] with 854 patients has demonstrated that the conversion rate to open surgery was significantly lower in the robotic approach when compared to the laparoscopic surgery (OR = 0.26; 95%CI: 0.12-0.57, P = 0.0007). In that study, there was no significant difference between the groups regarding operative length, hospital stay, postoperative complications, number of recovered lymph nodes and positive radial/distal margin status. In another study[90] including 84 patients who have undergone uLAR, the robotic surgery showed a lower conversion rate to the open procedure (robot, 2.1% *vs* laparoscopy, 16.2%, P = 0.02) and shorter hospital stay (robot, 9 d *vs* laparoscopy, 11 d, P = 0.011). There was no significant difference between the groups in rates of local recurrence and overall survival within 3 years. The robotic system has been improving rapidly and has been successfully used even in complex surgical procedures such as uLAR with CAA and ISR[91]. Even showing some potential advantages in relation to laparoscopy, the role of the robotic surgery for the rectal cancer treatment has not been defined yet, still requiring further studies with longer follow-up period.

CONCLUSION

Rectal cancer management is currently a multidisciplinary effort, which incorporates new concepts and technologies, resulting in significant improvement in patients' oncological and functional outcomes. Despite the evolution seen in the last decades, there are still many unanswered questions about the management of rectal cancer. We still await results of large multi-institutional prospective trials to define some of the most important and controversial points, such as safety and efficacy of the "wait and see" approach and the definitive role of laparoscopic and robotic surgery in rectal cancer.

REFERENCES

1 **Nivatvongs S**, Gordon PH. Surgical anatomy. In: Gordon PH, Nivatvongs S. Principle and Practice of Surgery for the Colon, Rectum and Anus. New York: Informa Healthcare, 2007: 1-28

2 **Monson JR**, Weiser MR, Buie WD, Chang GJ, Rafferty JF, Buie WD, Rafferty J; Standards Practice Task Force of the American Society of Colon and Rectal Surgeons. Practice parameters for the management of rectal cancer (revised). *Dis Colon Rectum* 2013; **56**: 535-550 [PMID: 23575392 DOI: 10.1097/DCR.0b013e31828cb66c]

3 **Kim MS**, Park YJ. Detection and treatment of synchronous lesions in colorectal cancer: the clinical implication of perioperative colonoscopy. *World J Gastroenterol* 2007; **13**: 4108-4111 [PMID: 17696231]

4 **AJCC**. Colon and Rectum. In: Edge SB, Byrd DR, Compton CC, eds. AJCC Cancer Staging Manual. 7th ed. New York, NY: Springer, 2010; 143-164

5 **Rifkin MD**, Ehrlich SM, Marks G. Staging of rectal carcinoma: prospective comparison of endorectal US and CT. *Radiology* 1989; **170**: 319-322 [PMID: 2643135]

6 **Shank B**, Dershaw DD, Caravelli J, Barth J, Enker W. A prospective study of the accuracy of preoperative computed tomographic staging of patients with biopsy-proven rectal carcinoma. *Dis Colon Rectum* 1990; **33**: 285-290 [PMID: 2323277 DOI: 10.1007/BF02055469]

7 **Goldman S**, Arvidsson H, Norming U, Lagerstedt U, Magnusson I, Frisell J. Transrectal ultrasound and computed tomography in preoperative staging of lower rectal adenocarcinoma. *Gastrointest Radiol* 1991; **16**: 259-263 [PMID: 1879647 DOI: 10.1007/BF01887361]

8 **Cova M**, Frezza F, Pozzi-Mucelli RS, Ukmar M, Tarjan Z, Melato M, Bucconi S, Dalla Palma L. Computed tomography and magnetic resonance in the preoperative staging of the spread of rectal cancer. A correlation with the anatomicopathological aspects. *Radiol Med* 1994; **87**: 82-89 [PMID: 8128038]

9 **Kim NK**, Kim MJ, Yun SH, Sohn SK, Min JS. Comparative study of transrectal ultrasonography, pelvic computerized tomography, and magnetic resonance imaging in preoperative staging of rectal cancer. *Dis Colon Rectum* 1999; **42**: 770-775 [PMID: 10378601 DOI: 10.1007/BF02236933]

10 **Muthusamy VR**, Chang KJ. Optimal methods for staging rectal cancer. *Clin Cancer Res* 2007; **13**: 6877s-6884s [PMID: 18006793 DOI: 10.1158/1078-0432.CCR-07-1137]

11 **Garcia-Aguilar J**, Pollack J, Lee SH, Hernandez de Anda E, Mellgren A, Wong WD, Finne CO, Rothenberger DA, Madoff RD. Accuracy of endorectal ultrasonography in preoperative staging of rectal tumors. *Dis Colon Rectum* 2002; **45**: 10-15 [PMID: 11786756 DOI: 10.1007/s10350-004-6106-3]

12 **Marusch F**, Koch A, Schmidt U, Zippel R, Kuhn R, Wolff S, Pross M, Wierth A, Gastinger I, Lippert H. Routine use of transrectal ultrasound in rectal carcinoma: results of a prospective multicenter study. *Endoscopy* 2002; **34**: 385-390 [PMID: 11972270 DOI: 10.1055/s-2002-25292]

13 **Dworák O**. Number and size of lymph nodes and node metastases in rectal carcinomas. *Surg Endosc* 1989; **3**: 96-99 [PMID: 2672395 DOI: 10.1007/BF00590909]

14 **Andreola S**, Leo E, Belli F, Bufalino R, Tomasic G, Lavarino C, Baldini MT, Meroni E. Manual dissection of adenocarcinoma of the lower third of the rectum specimens for detection of lymph node metastases smaller than 5 mm. *Cancer* 1996; **77**: 607-612 [PMID: 8616750]

15 **Bipat S**, Glas AS, Slors FJ, Zwinderman AH, Bossuyt PM,

Stoker J. Rectal cancer: local staging and assessment of lymph node involvement with endoluminal US, CT, and MR imaging--a meta-analysis. *Radiology* 2004; **232**: 773-783 [PMID: 15273331 DOI: 10.1148/radiol.2323031368]
16 **Bleday R**. Local excision of rectal cancer. *World J Surg* 1997; **21**: 706-714 [PMID: 9276701 DOI: 10.1007/s002689900295]
17 **Sengupta S**, Tjandra JJ. Local excision of rectal cancer: what is the evidence? *Dis Colon Rectum* 2001; **44**: 1345-1361 [PMID: 11584215 DOI: 10.1007/BF02234796]
18 **Meredith KL**, Hoffe SE, Shibata D. The multidisciplinary management of rectal cancer. *Surg Clin North Am* 2009; **89**: 177-215, ix-x [PMID: 19186236 DOI: 10.1016/j.suc.2008.09.021]
19 **Nastro P**, Beral D, Hartley J, Monson JR. Local excision of rectal cancer: review of literature. *Dig Surg* 2005; **22**: 6-15 [PMID: 15761225 DOI: 10.1159/000084345]
20 **Neary P**, Makin GB, White TJ, White E, Hartley J, MacDonald A, Lee PW, Monson JR. Transanal endoscopic microsurgery: a viable operative alternative in selected patients with rectal lesions. *Ann Surg Oncol* 2003; **10**: 1106-1111 [PMID: 14597451 DOI: 10.1245/ASO.2003.01.441]
21 **Gavagan JA**, Whiteford MH, Swanstrom LL. Full-thickness intraperitoneal excision by transanal endoscopic microsurgery does not increase short-term complications. *Am J Surg* 2004; **187**: 630-634 [PMID: 15135680 DOI: 10.1016/j.amjsurg.2004.01.004]
22 **Allaix ME**, Fichera A. Modern rectal cancer multidisciplinary treatment: the role of radiation and surgery. *Ann Surg Oncol* 2013; **20**: 2921-2928 [PMID: 23604783 DOI: 10.1245/s10434-013-2966-x]
23 **Påhlman L**, Glimelius B. Pre- or postoperative radiotherapy in rectal and rectosigmoid carcinoma. Report from a randomized multicenter trial. *Ann Surg* 1990; **211**: 187-195 [PMID: 2405793 DOI: 10.1097/00000658-199002000-00011]
24 **Cedermark B**, Johansson H, Rutqvist LE, Wilking N. The Stockholm I trial of preoperative short term radiotherapy in operable rectal carcinoma. A prospective randomized trial. Stockholm Colorectal Cancer Study Group. *Cancer* 1995; **75**: 2269-2275 [PMID: 7712435]
25 Improved survival with preoperative radiotherapy in resectable rectal cancer. Swedish Rectal Cancer Trial. *N Engl J Med* 1997; **336**: 980-987 [PMID: 9091798 DOI: 10.1056/NEJM199704033361402]
26 **Kapiteijn E**, Marijnen CA, Nagtegaal ID, Putter H, Steup WH, Wiggers T, Rutten HJ, Pahlman L, Glimelius B, van Krieken JH, Leer JW, van de Velde CJ; Dutch Colorectal Cancer Group. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. *N Engl J Med* 2001; **345**: 638-646 [PMID: 11547717 DOI: 10.1056/NEJMoa010580]
27 **Martling A**, Holm T, Johansson H, Rutqvist LE, Cedermark B; Stockholm Colorectal Cancer Study Group. The Stockholm II trial on preoperative radiotherapy in rectal carcinoma: long-term follow-up of a population-based study. *Cancer* 2001; **92**: 896-902 [PMID: 11550163]
28 **Sauer R**, Becker H, Hohenberger W, Rödel C, Wittekind C, Fietkau R, Martus P, Tschmelitsch J, Hager E, Hess CF, Karstens JH, Liersch T, Schmidberger H, Raab R; German Rectal Cancer Study Group. Preoperative versus postoperative chemoradiotherapy for rectal cancer. *N Engl J Med* 2004; **351**: 1731-1740 [PMID: 15496622 DOI: 10.1056/NEJMoa040694]
29 **Bujko K**, Nowacki MP, Nasierowska-Guttmejer A, Michalski W, Bebenek M, Kryj M. Long-term results of a randomized trial comparing preoperative short-course radiotherapy with preoperative conventionally fractionated chemoradiation for rectal cancer. *Br J Surg* 2006; **93**: 1215-1223 [PMID: 16983741 DOI: 10.1002/bjs.5506]
30 **Sebag-Montefiore D**, Stephens RJ, Steele R, Monson J, Grieve R, Khanna S, Quirke P, Couture J, de Metz C, Myint AS, Bessell E, Griffiths G, Thompson LC, Parmar M. Preoperative radiotherapy versus selective postoperative chemoradiotherapy in patients with rectal cancer (MRC CR07 and NCIC-CTG C016): a multicentre, randomised trial. *Lancet* 2009; **373**: 811-820 [PMID: 19269519 DOI: 10.1016/S0140-6736(09)60484-0]
31 **Sauer R**, Liersch T, Merkel S, Fietkau R, Hohenberger W, Hess C, Becker H, Raab HR, Villanueva MT, Witzigmann H, Wittekind C, Beissbarth T, Rödel C. Preoperative versus postoperative chemoradiotherapy for locally advanced rectal cancer: results of the German CAO/ARO/AIO-94 randomized phase III trial after a median follow-up of 11 years. *J Clin Oncol* 2012; **30**: 1926-1933 [PMID: 22529255 DOI: 10.1200/JCO.2011.40.1836]
32 **Rahbari NN**, Elbers H, Askoxylakis V, Motschall E, Bork U, Büchler MW, Weitz J, Koch M. Neoadjuvant radiotherapy for rectal cancer: meta-analysis of randomized controlled trials. *Ann Surg Oncol* 2013; **20**: 4169-4182 [PMID: 24002536 DOI: 10.1245/s10434-013-3198-9]
33 **Valentini V**, Coco C, Cellini N, Picciocchi A, Genovesi D, Mantini G, Barbaro B, Cogliandolo S, Mattana C, Ambesi-Impiombato F, Tedesco M, Cosimelli M. Preoperative chemoradiation for extraperitoneal T3 rectal cancer: acute toxicity, tumor response, and sphincter preservation. *Int J Radiat Oncol Biol Phys* 1998; **40**: 1067-1075 [PMID: 9539561 DOI: 10.1016/S0360-3016(97)00918-8]
34 **Gerard JP**, Rostom Y, Gal J, Benchimol D, Ortholan C, Aschele C, Levi JM. Can we increase the chance of sphincter saving surgery in rectal cancer with neoadjuvant treatments: lessons from a systematic review of recent randomized trials. *Crit Rev Oncol Hematol* 2012; **81**: 21-28 [PMID: 21377377 DOI: 10.1016/j.critrevonc.2011.02.001]
35 **Bujko K**, Kepka L, Michalski W, Nowacki MP. Does rectal cancer shrinkage induced by preoperative radio(chemo)therapy increase the likelihood of anterior resection? A systematic review of randomised trials. *Radiother Oncol* 2006; **80**: 4-12 [PMID: 16730086 DOI: 10.1016/j.critrevonc.2011.02.001]
36 **Glynne-Jones R**, Hughes R. Critical appraisal of the 'wait and see' approach in rectal cancer for clinical complete responders after chemoradiation. *Br J Surg* 2012; **99**: 897-909 [PMID: 22539154 DOI: 10.1002/bjs.8732]
37 **Martin ST**, Heneghan HM, Winter DC. Systematic review and meta-analysis of outcomes following pathological complete response to neoadjuvant chemoradiotherapy for rectal cancer. *Br J Surg* 2012; **99**: 918-928 [PMID: 22362002 DOI: 10.1002/bjs.8702]
38 **Habr-Gama A**, de Souza PM, Ribeiro U, Nadalin W, Gansl R, Sousa AH, Campos FG, Gama-Rodrigues J. Low rectal cancer: impact of radiation and chemotherapy on surgical treatment. *Dis Colon Rectum* 1998; **41**: 1087-1096 [PMID: 9749491 DOI: 10.1007/BF02239429]
39 **Habr-Gama A**, Perez RO, Proscurshim I, Campos FG, Nadalin W, Kiss D, Gama-Rodrigues J. Patterns of failure and survival for nonoperative treatment of stage c0 distal rectal cancer following neoadjuvant chemoradiation therapy. *J Gastrointest Surg* 2006; **10**: 1319-1328; discussion 1328-1329 [PMID: 17175450 DOI: 10.1016/j.gassur.2006.09.005]
40 **Nyasavajjala SM**, Shaw AG, Khan AQ, Brown SR, Lund JN. Neoadjuvant chemo-radiotherapy and rectal cancer: can the UK watch and wait with Brazil? *Colorectal Dis* 2010; **12**: 33-36 [PMID: 19832875 DOI: 10.1111/j.1463-1318.2009.02054.x]
41 **Nakagawa WT**, Rossi BM, de O Ferreira F, Ferrigno R, David Filho WJ, Nishimoto IN, Vieira RA, Lopes A. Chemoradiation instead of surgery to treat mid and low rectal tumors: is it safe? *Ann Surg Oncol* 2002; **9**: 568-573 [PMID: 12095973 DOI: 10.1007/BF02573893]
42 **Habr-Gama A**. Assessment and management of the complete clinical response of rectal cancer to chemoradiotherapy. *Colorectal Dis* 2006; **8** Suppl 3: 21-24 [PMID: 16813588 DOI: 10.1111/j.1463-1318.2006.01066.x]
43 **Lim L**, Chao M, Shapiro J, Millar JL, Kipp D, Rezo A, Fong

A, Jones IT, McLaughlin S, Gibbs P. Long-term outcomes of patients with localized rectal cancer treated with chemoradiation or radiotherapy alone because of medical inoperability or patient refusal. *Dis Colon Rectum* 2007; **50**: 2032-2039 [PMID: 17896138 DOI: 10.1007/s10350-007-9062-x]

44 **Hughes R**, Harrison M, Glynne-Jones R. Could a wait and see policy be justified in T3/4 rectal cancers after chemoradiotherapy? *Acta Oncol* 2010; **49**: 378-381 [PMID: 20151936 DOI: 10.3109/02841860903483692]

45 **Dalton RS**, Velineni R, Osborne ME, Thomas R, Harries S, Gee AS, Daniels IR. A single-centre experience of chemoradiotherapy for rectal cancer: is there potential for nonoperative management? *Colorectal Dis* 2012; **14**: 567-571 [PMID: 21831177 DOI: 10.1111/j.1463-1318.2011.02752.x]

46 **Maas M**, Beets-Tan RG, Lambregts DM, Lammering G, Nelemans PJ, Engelen SM, van Dam RM, Jansen RL, Sosef M, Leijtens JW, Hulsewé KW, Buijsen J, Beets GL. Wait-and-see policy for clinical complete responders after chemoradiation for rectal cancer. *J Clin Oncol* 2011; **29**: 4633-4640 [PMID: 22067400 DOI: 10.1200/JCO.2011.37.7176]

47 **Evans J**, Patel U, Brown G. Rectal cancer: primary staging and assessment after chemoradiotherapy. *Semin Radiat Oncol* 2011; **21**: 169-177 [PMID: 21645861 DOI: 10.1016/j.semradonc.2011.02.002]

48 **Mekras A**, Michalopoulos A, Papadopoulos VN, Mekras D, Kalles V, Tzeveleki I, Dabakis G, Netta S, Basdanis G. Changes in treatment of rectal cancer: increased use of low anterior resection. *Tech Coloproctol* 2011; **15** Suppl 1: S51-S54 [PMID: 21887575 DOI: 10.1007/s10151-011-0731-3]

49 **Wibe A**, Syse A, Andersen E, Tretli S, Myrvold HE, Søreide O; Norwegian Rectal Cancer Group. Oncological outcomes after total mesorectal excision for cure for cancer of the lower rectum: anterior vs. abdominoperineal resection. *Dis Colon Rectum* 2004; **47**: 48-58 [PMID: 14719151 DOI: 10.1007/s10350-003-0012-y]

50 **McCall JL**, Cox MR, Wattchow DA. Analysis of local recurrence rates after surgery alone for rectal cancer. *Int J Colorectal Dis* 1995; **10**: 126-132 [PMID: 7561427 DOI: 10.1007/BF00298532]

51 **Tan WS**, Tang CL, Shi L, Eu KW. Meta-analysis of defunctioning stomas in low anterior resection for rectal cancer. *Br J Surg* 2009; **96**: 462-472 [PMID: 19358171 DOI: 10.1002/bjs.6594]

52 **Gessler B**, Haglind E, Angenete E. Loop ileostomies in colorectal cancer patients--morbidity and risk factors for nonreversal. *J Surg Res* 2012; **178**: 708-714 [PMID: 22940030 DOI: 10.1016/j.jss.2012.08.018]

53 **Rutkowski A**, Bujko K, Nowacki MP, Chmielik E, Nasierowska-Guttmejer A, Wojnar A; Polish Colorectal Study Group. Distal bowel surgical margin shorter than 1 cm after preoperative radiation for rectal cancer: is it safe? *Ann Surg Oncol* 2008; **15**: 3124-3131 [PMID: 18766404 DOI: 10.1245/s10434-008-0125-6]

54 **Moore HG**, Riedel E, Minsky BD, Saltz L, Paty P, Wong D, Cohen AM, Guillem JG. Adequacy of 1-cm distal margin after restorative rectal cancer resection with sharp mesorectal excision and preoperative combined-modality therapy. *Ann Surg Oncol* 2003; **10**: 80-85 [PMID: 12513965 DOI: 10.1245/ASO.2003.04.010]

55 **Guillem JG**, Chessin DB, Shia J, Suriawinata A, Riedel E, Moore HG, Minsky BD, Wong WD. A prospective pathologic analysis using whole-mount sections of rectal cancer following preoperative combined modality therapy: implications for sphincter preservation. *Ann Surg* 2007; **245**: 88-93 [PMID: 17197970 DOI: 10.1097/01.sla.0000232540.82364.43]

56 **Nelson H**, Petrelli N, Carlin A, Couture J, Fleshman J, Guillem J, Miedema B, Ota D, Sargent D; National Cancer Institute Expert Panel. Guidelines 2000 for colon and rectal cancer surgery. *J Natl Cancer Inst* 2001; **93**: 583-596 [PMID: 11309435 DOI: 10.1093/jnci/93.8.583]

57 **Wolmark N**, Fisher B. An analysis of survival and treatment failure following abdominoperineal and sphincter-saving resection in Dukes' B and C rectal carcinoma. A report of the NSABP clinical trials. National Surgical Adjuvant Breast and Bowel Project. *Ann Surg* 1986; **204**: 480-489 [PMID: 3532972 DOI: 10.1097/00000658-198610000-00016]

58 **Williams NS**, Dixon MF, Johnston D. Reappraisal of the 5 centimetre rule of distal excision for carcinoma of the rectum: a study of distal intramural spread and of patients' survival. *Br J Surg* 1983; **70**: 150-154 [PMID: 6831156 DOI: 10.1002/bjs.1800700305]

59 **Ueno H**, Mochizuki H, Hashiguchi Y, Ishikawa K, Fujimoto H, Shinto E, Hase K. Preoperative parameters expanding the indication of sphincter preserving surgery in patients with advanced low rectal cancer. *Ann Surg* 2004; **239**: 34-42 [PMID: 14685098 DOI: 10.1097/01.sla.0000103070.13030.eb]

60 **Bujko K**, Rutkowski A, Chang GJ, Michalski W, Chmielik E, Kusnierz J. Is the 1-cm Rule of Distal Bowel Resection Margin in Rectal Cancer Based on Clinical Evidence? A Systematic Review. *Indian J Surg Oncol* 2012; **3**: 139-146 [PMID: 23728157 DOI: 10.1007/s13193-012-0158-y]

61 **Heald RJ**, Husband EM, Ryall RD. The mesorectum in rectal cancer surgery--the clue to pelvic recurrence? *Br J Surg* 1982; **69**: 613-616 [PMID: 6751457 DOI: 10.1002/bjs.1800691019]

62 **Scott N**, Jackson P, al-Jaberi T, Dixon MF, Quirke P, Finan PJ. Total mesorectal excision and local recurrence: a study of tumour spread in the mesorectum distal to rectal cancer. *Br J Surg* 1995; **82**: 1031-1033 [PMID: 7648142 DOI: 10.1002/bjs.1800820808]

63 **Hida J**, Yasutomi M, Maruyama T, Fujimoto K, Uchida T, Okuno K. Lymph node metastases detected in the mesorectum distal to carcinoma of the rectum by the clearing method: justification of total mesorectal excision. *J Am Coll Surg* 1997; **184**: 584-588 [PMID: 9179114]

64 **Quirke P**, Durdey P, Dixon MF, Williams NS. Local recurrence of rectal adenocarcinoma due to inadequate surgical resection. Histopathological study of lateral tumour spread and surgical excision. *Lancet* 1986; **2**: 996-999 [PMID: 2430152 DOI: 10.1016/S0140-6736(86)92612-7]

65 **Adam IJ**, Mohamdee MO, Martin IG, Scott N, Finan PJ, Johnston D, Dixon MF, Quirke P. Role of circumferential margin involvement in the local recurrence of rectal cancer. *Lancet* 1994; **344**: 707-711 [PMID: 7915774 DOI: 10.1016/S0140-6736(94)92206-3]

66 **Enker WE**. Total mesorectal excision--the new golden standard of surgery for rectal cancer. *Ann Med* 1997; **29**: 127-133 [PMID: 9187227 DOI: 10.3109/07853899709113698]

67 **Saito N**, Ito M, Kobayashi A, Nishizawa Y, Sugito M. Sphincter-saving resection for low rectal cancer. *Nihon Geka Gakkai Zasshi* 2011; **112**: 318-324 [PMID: 21941822]

68 **Rullier E**, Denost Q, Vendrely V, Rullier A, Laurent C. Low rectal cancer: classification and standardization of surgery. *Dis Colon Rectum* 2013; **56**: 560-567 [PMID: 23575394 DOI: 10.1097/DCR.0b013e31827c4a8c]

69 **Grumann MM**, Noack EM, Hoffmann IA, Schlag PM. Comparison of quality of life in patients undergoing abdominoperineal extirpation or anterior resection for rectal cancer. *Ann Surg* 2001; **233**: 149-156 [PMID: 11176118 DOI: 10.1097/00000658-200102000-00001]

70 **Chin CC**, Yeh CY, Huang WS, Wang JY. Clinical outcome of intersphincteric resection for ultra-low rectal cancer. *World J Gastroenterol* 2006; **12**: 640-643 [PMID: 16489683]

71 **Gamagami R**, Istvan G, Cabarrot P, Liagre A, Chiotasso P, Lazorthes F. Fecal continence following partial resection of the anal canal in distal rectal cancer: long-term results after coloanal anastomoses. *Surgery* 2000; **127**: 291-295 [PMID: 10715984 DOI: 10.1067/msy.2000.103487]

72 **Rosen L**, Stasik JJ, Reed JF, Olenwine JA, Aronoff JS, Sherman D. Variations in colon and rectal surgical mortality. Comparison of specialties with a state-legislated database.

Dis Colon Rectum 1996; **39**: 129-135 [PMID: 8620777 DOI: 10.1007/BF02068065]
73 **Borowski DW**, Kelly SB, Bradburn DM, Wilson RG, Gunn A, Ratcliffe AA; Northern Region Colorectal Cancer Audit Group. Impact of surgeon volume and specialization on short-term outcomes in colorectal cancer surgery. *Br J Surg* 2007; **94**: 880-889 [PMID: 17410637 DOI: 10.1002/bjs.5721]
74 **Porter GA**, Soskolne CL, Yakimets WW, Newman SC. Surgeon-related factors and outcome in rectal cancer. *Ann Surg* 1998; **227**: 157-167 [PMID: 9488510 DOI: 10.1097/00000658-199802000-00001]
75 **Archampong D**, Borowski D, Wille-Jørgensen P, Iversen LH. Workload and surgeon's specialty for outcome after colorectal cancer surgery. *Cochrane Database Syst Rev* 2012; **3**: CD005391 [PMID: 22419309 DOI: 10.1002/14651858]
76 **Read TE**, Myerson RJ, Fleshman JW, Fry RD, Birnbaum EH, Walz BJ, Kodner IJ. Surgeon specialty is associated with outcome in rectal cancer treatment. *Dis Colon Rectum* 2002; **45**: 904-914 [PMID: 12130879 DOI: 10.1007/s10350-004-6327-5]
77 **Jayne DG**, Guillou PJ, Thorpe H, Quirke P, Copeland J, Smith AM, Heath RM, Brown JM; UK MRC CLASICC Trial Group. Randomized trial of laparoscopic-assisted resection of colorectal carcinoma: 3-year results of the UK MRC CLASICC Trial Group. *J Clin Oncol* 2007; **25**: 3061-3068 [PMID: 17634484 DOI: 10.1200/JCO.2006.09.7758]
78 **Braga M**, Frasson M, Vignali A, Zuliani W, Capretti G, Di Carlo V. Laparoscopic resection in rectal cancer patients: outcome and cost-benefit analysis. *Dis Colon Rectum* 2007; **50**: 464-471 [PMID: 17195085 DOI: 10.1007/s10350-006-0798-5]
79 **Hillingsø JG**, Wille-Jørgensen P. Staged or simultaneous resection of synchronous liver metastases from colorectal cancer--a systematic review. *Colorectal Dis* 2009; **11**: 3-10 [PMID: 18637099 DOI: 10.1111/j.1463-1318.2008.01625.x]
80 **Laurent C**, Leblanc F, Wütrich P, Scheffler M, Rullier E. Laparoscopic versus open surgery for rectal cancer: long-term oncologic results. *Ann Surg* 2009; **250**: 54-61 [PMID: 19561481 DOI: 10.1097/SLA.0b013e3181ad6511]
81 **Kang SB**, Park JW, Jeong SY, Nam BH, Choi HS, Kim DW, Lim SB, Lee TG, Kim DY, Kim JS, Chang HJ, Lee HS, Kim SY, Jung KH, Hong YS, Kim JH, Sohn DK, Kim DH, Oh JH. Open versus laparoscopic surgery for mid or low rectal cancer after neoadjuvant chemoradiotherapy (COREAN trial): short-term outcomes of an open-label randomised controlled trial. *Lancet Oncol* 2010; **11**: 637-645 [PMID: 20610322 DOI: 10.1016/S1470-2045(10)70131-5]
82 **Anderson C**, Uman G, Pigazzi A. Oncologic outcomes of laparoscopic surgery for rectal cancer: a systematic review and meta-analysis of the literature. *Eur J Surg Oncol* 2008; **34**: 1135-1142 [PMID: 18191529 DOI: 10.1016/j.ejso.2007.11.015]
83 **Jayne DG**, Thorpe HC, Copeland J, Quirke P, Brown JM, Guillou PJ. Five-year follow-up of the Medical Research Council CLASICC trial of laparoscopically assisted versus open surgery for colorectal cancer. *Br J Surg* 2010; **97**: 1638-1645 [PMID: 20629110 DOI: 10.1002/bjs.7160]
84 **van der Pas MH**, Haglind E, Cuesta MA, Fürst A, Lacy AM, Hop WC, Bonjer HJ. Laparoscopic versus open surgery for rectal cancer (COLOR II): short-term outcomes of a randomised, phase 3 trial. *Lancet Oncol* 2013; **14**: 210-218 [PMID: 23395398 DOI: 10.1016/S1470-2045(13)70016-0]
85 **Fleshman J**; American College of Surgeons Oncology Group (ACOSOG)-Z6051. A Phase III prospective randomized trial comparing laparoscopic-assisted resection vs open resection for rectal cancer. Available from: URL: http://clinicaltrials.gov/ct2/show. Accessed on september 23, 2013
86 **Lanfranco AR**, Castellanos AE, Desai JP, Meyers WC. Robotic surgery: a current perspective. *Ann Surg* 2004; **239**: 14-21 [PMID: 14685095 DOI: 10.1097/01.sla.0000103020.19595.7d]
87 **Alasari S**, Min BS. Robotic colorectal surgery: a systematic review. *ISRN Surg* 2012; **2012**: 293894 [PMID: 22655207 DOI: 10.5402/2012/293894]
88 **Aly EH**. Robotic colorectal surgery: summary of the current evidence. *Int J Colorectal Dis* 2014; **29**: 1-8 [PMID: 23995270]
89 **Trastulli S**, Farinella E, Cirocchi R, Cavaliere D, Avenia N, Sciannameo F, Gullà N, Noya G, Boselli C. Robotic resection compared with laparoscopic rectal resection for cancer: systematic review and meta-analysis of short-term outcome. *Colorectal Dis* 2012; **14**: e134-e156 [PMID: 22151033 DOI: 10.1111/j.1463-1318.2011.02907.x]
90 **Baek SJ**, Al-Asari S, Jeong DH, Hur H, Min BS, Baik SH, Kim NK. Robotic versus laparoscopic coloanal anastomosis with or without intersphincteric resection for rectal cancer. *Surg Endosc* 2013; **27**: 4157-4163 [PMID: 23708725]
91 **Kang J**, Hur H, Min BS, Lee KY, Kim NK. Robotic coloanal anastomosis with or without intersphincteric resection for low rectal cancer: starting with the perianal approach followed by robotic procedure. *Ann Surg Oncol* 2012; **19**: 154-155 [PMID: 21822556 DOI: 10.1245/s10434-011-1952-4]

P- Reviewers: Altomare DF, Kim YJ, Vizoso FJ, Zielinski J
S- Editor: Gou SX **L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.888

World J Gastroenterol 2014 January 28; 20(4): 888-898
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Colorectal cancer biomarkers: To be or not to be? Cautionary tales from a road well travelled

Kim YC Fung, Edouard Nice, Ilka Priebe, Damien Belobrajdic, Aloke Phatak, Leanne Purins, Bruce Tabor, Celine Pompeia, Trevor Lockett, Timothy E Adams, Antony Burgess, Leah Cosgrove

Kim YC Fung, Ilka Priebe, Damien Belobrajdic, Leanne Purins, Bruce Tabor, Celine Pompeia, Trevor Lockett, Leah Cosgrove, CSIRO, Preventative Health National Research Flagship, Adelaide, SA 5000, Australia
Edouard Nice, Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC 3168, Australia
Aloke Phatak, CSIRO, Computational Informatics, Floreat, WA 6014, Australia
Timothy E Adams, CSIRO, Materials Science and Engineering, Parkville, VIC 3052, Australia
Antony Burgess, Walter and Eliza Hall Institute of Medical Research, Parkville, VIC 3052, Australia
Author contributions: Fung KYC and Nice E wrote the paper; Priebe I performed the research and analysed the data; Fung KYC, Nice E and Priebe I contributed equally to the work; Belobrajdic D contributed data to the study; Fung KYC, Phatak A, Purins L, Tabor B and Pompeia C analysed the data; Lockett T, Adams TE, Burgess A and Cosgrove L designed the research.
Supported by The CSIRO Preventative Health National Research Flagship and the National Health and Medical Research Council, No. 1017078
Correspondence to: Kim YC Fung, PhD, CSIRO, Preventative Health National Research Flagship, PO Box 10041, Adelaide, SA 5000, Australia. kim.fung@csiro.au
Telephone: +61-8-83038840 Fax: +61-8-83038899
Received: September 26, 2013 Revised: November 26, 2013
Accepted: January 6, 2014
Published online: January 28, 2014

Abstract

Colorectal cancer (CRC) is the second most common cause of cancer-related death worldwide and places a major economic burden on the global health care system. The time frame for development from premalignant to malignant disease typically spans 10-15 years, and this latent period provides an ideal opportunity for early detection and intervention to improve patient outcomes. Currently, early diagnosis of CRC is hampered by a lack of suitable non-invasive biomarkers that are clinically or economically acceptable for population-based screening. New blood-based protein biomarkers for early detection of CRC are therefore urgently required. The success of clinical biomarker discovery and validation studies is critically dependent on understanding and adjusting for potential experimental, analytical, and biological factors that can interfere with the robust interpretation of results. In this review we outline some important considerations for research groups undertaking biomarker research with exemplars from our studies. Implementation of experimental strategies to minimise the potential effects of these problems will facilitate the identification of panels of biomarkers with the sensitivity and specificity required for the development of successful tests for the early detection and surveillance of CRC.

Key words: Biomarker; Bias; Colorectal cancer; Diagnostic; Discovery; Validation

Core tip: The identification of sensitive and specific biomarkers for the early diagnosis and surveillance of colorectal cancer is recognised as being fundamental to improve survival for this disease. Studies involving analyses of multiple biomarkers require consideration of many potential confounding issues, some of which are impossible or difficult to control for. Implementation of strategies which can overcome and account for potentially confounding variables is essential to ensure robust verification and validation of potential biomarkers and their successful evaluation in large and meaningful clinical cohorts that are representative of the target population, ultimately with successful translation into the clinic.

Fung KYC, Nice E, Priebe I, Belobrajdic D, Phatak A, Purins L, Tabor B, Pompeia C, Lockett T, Adams TE, Burgess A, Cosgrove L. Colorectal cancer biomarkers: To be or not to be? Cautionary tales from a road well travelled. *World J Gastroenterol* 2014; 20(4): 888-898 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/888.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.888

INTRODUCTION

Worldwide, colorectal cancer (CRC) is one of the most prevalent cancers representing approximately 10% of all cancer diagnoses[1]. This places a major economic burden on the global health care system[2]. CRC is, however, regarded as one of the most preventable diseases as lifestyle and diet are believed to be major causative factors in disease development[3]. Epidemiological studies have shown that smoking, excess body weight, physical inactivity and low consumption of dietary fibre are risk factors for CRC[3]. Early detection of CRC is especially important as patients who are diagnosed early (TNM Stage I disease) have a 5-year survival rate of 90%-95% following surgical resection[4]. In contrast, when diagnosed at the later stages (*i.e.*, Stage Ⅳ), the 5-year survival rate is only 5%-10%. Currently, the faecal occult blood test (FOBT) and faecal immune test are the only clinically accepted non-invasive diagnostic tests for CRC[5]. These tests detect the presence of haem or blood in stool, but have low sensitivity for CRC (61%-79% sensitivity at 86%-95% specificity)[6-8] and perform poorly for early disease detection (sensitivity of 27% and 50% for advanced neoplasia and Dukes Stage A, respectively)[7]. While colonoscopy and sigmoidoscopy have high specificity for CRC and are capable of early detection, they are highly invasive and costly procedures. Early stages of the disease (premalignant or Stage 1) are asymptomatic and it is estimated that up to 50% of patients already have invasive cancer or metastasis at presentation. Consequently, to reduce mortality from this disease, an improved sensitive and specific non-invasive screening test for CRC is urgently needed.

Early diagnosis, including detection of adenomas, is considered to be a key aspect for improving patient survival and prognostic or predictive biomarkers are essential for guiding patient therapy or monitoring treatment efficacy. However, the success of biomarker translation into the clinic has been limited and very few biomarkers have passed the steps necessary for routine clinical utility. The US Food and Drug Administration (FDA) has approved less than 30 cancer biomarkers, primarily to monitor response to therapy, over recent years despite the thousands of research papers published every year[9]. As yet, a diagnostic panel has not been identified for CRC despite extensive research efforts and numerous reports of potential multi-marker protein panels or gene signatures. These include multiple gene biomarker panels[10-15], individual protein biomarkers[16-19], metabolic markers[20], a stool DNA test[21], and the DNA methylation marker, septin 9 (mSEPT9)[22]. The most promising test to date is a stool DNA test comprised of a panel of four methylated genes (BMP3, NDRG4, vimentin, TFPI2), a mutant form of KRAS and α-actin as the internal reference control[21]. In a recent blinded multicentre trial, this panel was able to accurately detect Stage Ⅰ-Ⅲ CRC patients with 87% sensitivity at 90% specificity in a training set and with 78% sensitivity at 85% specificity in a test set (combined sensitivity of 85% at 90% specificity). More importantly, this test was also able to detect large polyps with a detection rate of 54% and 92% for polyps ⩾ 1 cm and > 4 cm, respectively. This test is currently awaiting FDA approval.

Recently, mSEPT9 has emerged as a promising diagnostic marker for CRC[22-25]. mSEPT measured in plasma is reported to have higher sensitivity and specificity than either the guaiac faecal occult blood test (gFOBT) or carcinoembryonic antigen (CEA)[24]. Tóth *et al*[24] reported a sensitivity of 79.3% for mSEPT9 *vs* 68.2% and 51.8% for gFOBT and CEA, respectively (specificity of 84.8%, 70.6% and 85.2%, respectively). Warren *et al*[25] also recently reported 90% sensitivity at 88% specificity for all disease stages for mSEPT9, 87% for Stage Ⅰ-Ⅱ disease and a detection rate of 12% for adenomas. Based on these studies, a prospective study was conducted in an asymptomatic screening population aged 50 years and older and this study determined that the sensitivity for mSEPT9 was 48% at 91% specificity, indicating that performance of this test in a screening population may not be optimal[26]. Furthermore, when compared with the stool DNA test mentioned above, the sensitivity for CRC was 87% for the stool DNA panel *vs* 60% for plasma mSEPT9 and the authors also reported that the stool DNA test was markedly more sensitive for early stage disease and proximal cancers than mSEPT9[27]. Although mSEPT9 is considered highly promising, a recent cost-effectiveness study conducted by Ladabaum *et al*[28] revealed that current established screening modalities were still more effective than mSEPT9 and that testing of mSEPT9 yielded only an incremental benefit. This study highlights that in addition to high sensitivity and specificity, a diagnostic test must fulfil additional criteria to be successfully adopted by the community.

Amongst the many proteins that have been proposed as potential diagnostic biomarkers for CRC, two protein biomarkers have been extensively investigated: the tumour specific M2 isoform of pyruvate kinase (PKM2) and tissue inhibitor of matrix metalloproteinase 1 (TIMP1). PKM2 measured in plasma and stool show relatively high sensitivity for CRC diagnosis, with reported sensitivity of over 90% in stool in some studies[29-31]. Plasma TIMP1 is reportedly elevated in CRC in comparison to control populations, and prospective studies have been conducted to determine its utility as a biomarker for CRC[32,33] based on published data of retrospective studies reporting sensitivity and specificity of TIMP1 of 63% at 98%, respectively, for CRC overall, and 56% sensitivity for early stage disease (Dukes Stages A and B)[34]. The re-

sults of prospective studies to date have been disappointing. Based on the results of the recent study by Neilson *et al*[33] which included 4509 individuals who undertook sigmoidoscopy or colonoscopy, TIMP1 measured in plasma was not demonstrably better than CEA at detecting CRC. Another prospective study by the same group also determined that no difference in plasma TIMP1 levels was detectable between patients with adenomas, polyps or no colon pathology, indicating that TIMP1 is not suitable for detection of premalignant lesions[35]. Accordingly, plasma TIMP1 is believed to be more sensitive for late stage disease (Stage D) in comparison to Stages A, B or C, and higher pre-operative levels are associated with poor prognosis[36-39]. Additionally, when compared to FOBT, both PKM2 and TIMP1 are less sensitive for disease detection[18,40].

CEA measured in serum and carbohydrate antigen 19-9 (CA19-9), a gastrointestinal tumour marker, are two well documented blood-based protein biomarkers used for cancer detection[41,42]. Serum CEA is widely used as a cancer biomarker to monitor recurrence, however, it is not recommended for use as a diagnostic marker as it is not specific for CRC or cancer, can be elevated in response to other physiological conditions, and has low sensitivity for diagnosis of CRC[43]. The sensitivity of CEA for early stage disease is relatively low and is higher in the later stages of disease. Wang *et al*[44] reported elevated pre-operative CEA levels in less than 40% of patients diagnosed with Stage A and B disease, and in 70% of patients with Stage C disease. Similar to CEA, CA19-9 is non-specific for cancer and elevated levels are detected in benign inflammatory diseases, especially benign intestinal and liver disease[45]. The measurement of CA19-9 in serum has lower sensitivity than CEA for CRC diagnosis and like CEA, its greatest clinical utility is to monitor disease progression and prognosis once cancer has been diagnosed[43-47].

For detection of disease recurrence, genomic signatures have been most successful, *e.g.*, MammaPrint, a 70-gene panel, has been approved by the US FDA as an in vitro diagnostic platform for breast cancer[48,49]. The clinical performance of platforms based on gene transcript signatures is still being evaluated for detection of recurrence for CRC but these appear to hold better promise as a stratification tool for Stage Ⅱ or Ⅲ CRC patients to determine those who are most likely to benefit from chemotherapy[10,12,14,50,51]. ColoGuideEx, a 13-gene classifier that appears more promising for stratification of Stage Ⅱ patients and ColoGuidePro, which utilises the expression of 7 genes to predict prognosis of Stage Ⅲ patients, are still in the research phase[10,51]. OncotypeDx, available commercially but as yet not assessed for clinical utility, is a 7-gene classifier developed from analysis of paraffin embedded CRC tissue[50] and ColoPrint, a test based on an 18-gene classifier in fresh frozen tissue, is currently recruiting patients for a Stage Ⅲ clinical trial[12,14].

Identification of novel biomarkers requires knowledge of disease heterogeneity and pathophysiology and basic research is initially required to determine if specific biomolecules are differentially expressed between disease and non-disease tissues/biofluids. The ready availability of sequencing and array technologies (*e.g.*, for DNA and RNA) and proteomic platforms enables many potential biomarker candidates to be identified using small numbers of samples and/or patients. Accordingly, once potential biomarkers are identified, robust validation studies on independent cohorts need to be performed to ensure only relevant biomarkers are carried forward into larger and more extensive case controlled studies using well-characterised cohorts. At this stage of the pipeline, major challenges remain where many factors need to be considered to determine the likely clinical success of candidate biomarkers including analytical variables, biomarker and biological variables and cohort composition (Table 1). It has been recognised that bias can be easily introduced in these early stages of the pipeline that may overestimate the likely performance of the biomarker being investigated[52]. Other factors to consider include invasiveness of the test, privacy, patient compliance and cost.

Table 1 Factors that can affect the outcome of biomarker studies

Analytical variables	Use of standard operating procedures for sample collection and processing
	Sample storage conditions (*e.g.*, liquid nitrogen, -80 ℃, aliquot size)
	Assay performance and reproducibility
Biomarker/ biological variables	Biomarker stability (*e.g.*, over time, under different storage conditions)
	Diurnal variation, fasting vs non-fasting
	Comorbidities, medications, diet
	Variability within a normal population
Cohort composition	Number of patients
	Inclusion/exclusion criteria for controls and patient selection
	Cohort balancing (*e.g.*, age, gender matching)

In this review, we provide examples from initial pilot and case-controlled studies we have conducted as part of our efforts to identify novel blood-based protein biomarkers for CRC diagnosis. Our primary objective is to define a panel of protein biomarkers in blood, with better specificity and selectivity than the current FOBT, that can be used in a non-invasive test to diagnose early stage CRC. Additionally, the number of unnecessary colonoscopies currently being performed due to false positive results would be greatly reduced. We will use data for two potential protein biomarkers for CRC [insulin-like growth factor binding protein 2 (IGFBP2) and matrix metalloproteinase 9 (MMP9)] to demonstrate the potential impact of experimental, analytical and biological variables on the interpretation of biomarker results.

BIOMARKER STABILITY UNDER DIFFERENT STORAGE CONDITIONS

Sample collection, processing and storage of clinical samples have been identified as a potential source of bias that can confound the results of biomarker studies[53-56].

Although it is impossible to control for all variables in these procedures, standard operating procedures are absolutely essential to standardise sample collection and processing[55]. As part of our studies we have implemented stringent standard operating procedures for sample collection, processing and storage[57,58], based on the Human Proteome Organisation and Early Detection Research Network guidelines[54,55]. In many cases, decisions regarding storage conditions, however, are usually based on practical considerations such as cost, type of collection (*i.e.*, retrospective or prospective collection), number of patient samples and laboratory facilities available. For biomarker studies, patient samples are typically collected and stored for a period of time (months or years) prior to analysis. In addition to collection and handling procedures, possible degradation of biomarkers over time due to factors such as storage conditions, aliquot size, or freeze/thaw cycles need to be considered. This is particularly important when measuring proteins that are present at low abundance in biological fluids and to ensure that experimental artefacts are not erroneously reported as specific to the disease[59]. Despite the general awareness of the potential impact of these confounders, there are few case-controlled protein biomarker studies reported in the literature that include assessment of these factors. Although necessary to ensure that the integrity of the protein is maintained, these studies are difficult to perform due to resource limitations and because each protein must be assessed independently due to the unique physiochemical properties of each protein that will govern its interactions with other biomolecules or surfaces, and affect its stability in biological matrices.

As part of our procedures, we determined the most suitable sample matrix for each biomarker (*i.e.*, serum or plasma) based on the literature, manufacturers recommendations and our own preliminary investigations. We also assessed the stability of the biomarkers over an 18 mo period when stored in either liquid nitrogen or at -80 ℃. Data for MMP9 and IGFBP2 are shown as exemplars (Figure 1). Protein levels in clinical samples were quantified using commercially available enzyme linked immunosorbent assay (ELISA) kits or reagents according to the manufacturers' instructions. IGFBP2 was measured using ELISA kits from DSL Inc. (Texas, United States) or Mediagnost (Kiel, Germany). MMP9 was measured using ELISA kits purchased from Quantikine (R&D Systems, Minneapolis, United States). The Prism software package (version 5.04, Graphpad Software Inc., San Diego, United States) was used for statistical analysis.

Figure 1 shows the stability of IGFBP2 and MMP9 (n = 10 patients) following 18 months storage at both -80 ℃ and in liquid nitrogen and the effect of multiple freeze/thaw cycles (n = 3). The assays themselves proved remarkably stable over this time period (Figure 1A). The concentration of IGFBP2 in serum samples was found to be stable over 18 mo, regardless of the storage conditions used (Figure 1B). The concentration of MMP9, however, decreased significantly when stored in liquid nitrogen, both overnight (13% ± 2%) and after 18 months (16% ± 3%) when compared to MMP9 measured immediately ($P < 0.05$) (Figure 1B). Both markers were found to be stable over multiple freeze/thaw cycles (Figure 1C). This suggests that for accurate measurement of MMP9, samples should be stored at -80 ℃ as storage in liquid nitrogen, both short and long term, resulted in protein losses.

We also determined the stability of markers in plasma or serum when using alternative collection tubes, to investigate possible losses by non-specific binding. Figure 2 shows the effect of these variables on IGFBP2 measurements. These data showed that IGFBP2 is best measured in serum following collection into serum separator tubes as this resulted in consistent measurements and also provided the highest yields for this biomarker. When measured in plasma with collection into either EDTA or citrate, IGFBP2 levels were significantly lower. Furthermore, this trend was consistent when IGFBP2 was measured immediately, or following overnight storage at 4 ℃ and -80 ℃ (Figure 2).

Whilst actual clinical measurements are typically made on fresh samples soon after collection, biomarker stability under different sample collection and storage conditions is becoming increasingly important as large multi-site and/or multi-institutional specimen biobanks are established as a resource for the scientific community for discovery and evaluation of biomarkers[60,61]. These biobanks have been established with the intention of providing very large numbers of biospecimens (from > 100000 participants) which have been collected under stringent standard operating protocols and that are well-characterised in terms of clinical data and patient history with the potential for obtaining follow-up information for prospective studies[60,61]. The primary rationale is that the performance of biomarkers identified by different research groups can be directly compared using a standard reference set to eliminate variability associated with sample collection, handling and storage procedures and to facilitate clinical and translational research. Although these resources are clearly valuable, researchers must also use caution when accessing these samples. For example, based on our initial investigations, analysis of IGFBP2 in serum is preferred while degradation of MMP9 following short or long term storage in liquid nitrogen indicates that sourcing samples from biobanks such as the European Prospective Investigation into Cancer and Nutrition Biobank for further validation studies might be less appropriate as these blood samples (plasma and serum) are stored in liquid nitrogen[61].

COHORT COMPOSITION AND THE "CONTROL" POPULATION

Biomarker studies are often case controlled studies that compare the concentrations of an analyte(s) in non-diseased (*i.e.*, normal or control) *vs* diseased populations. Clearly the results must be reproducible across indepen-

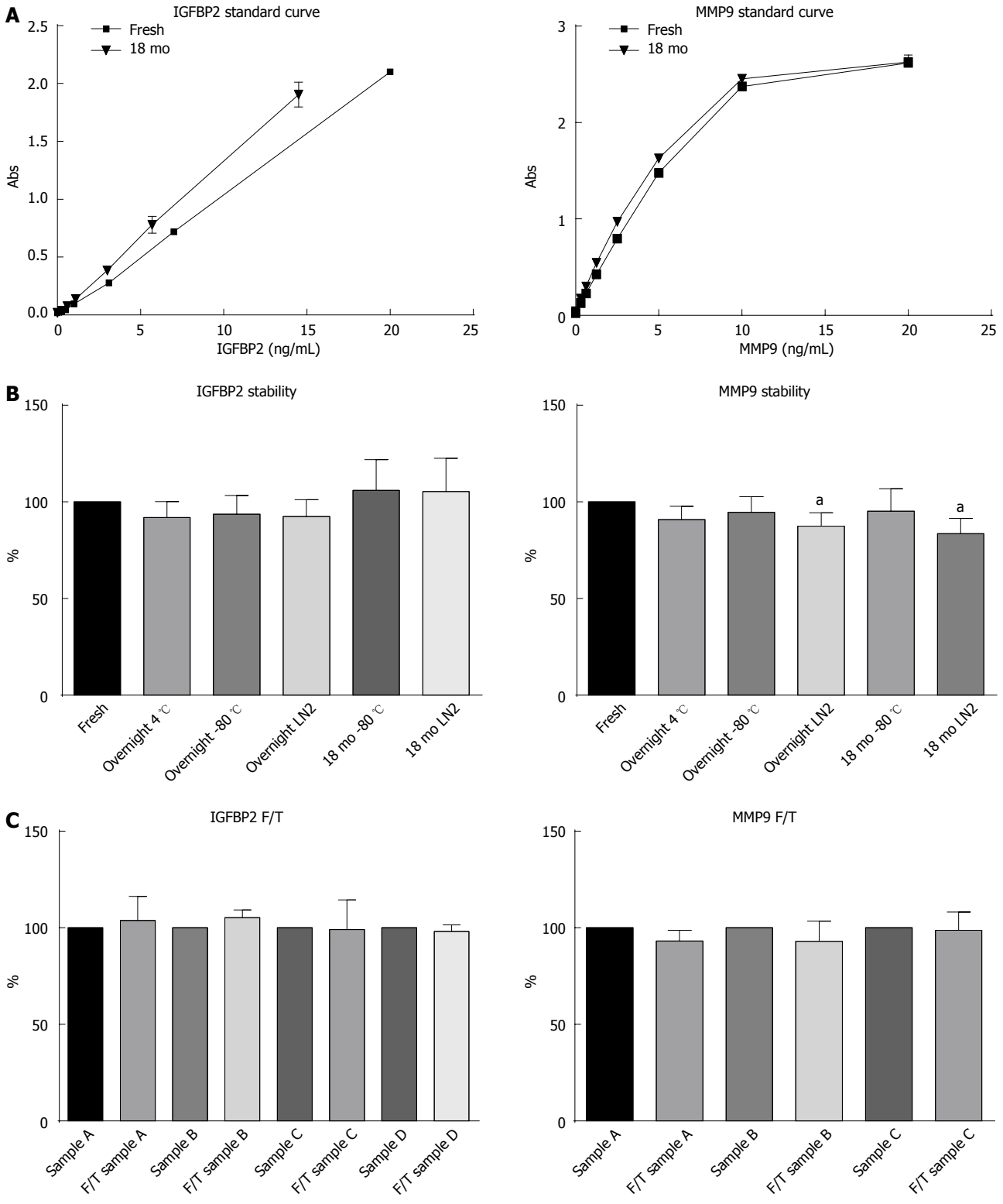


Figure 1 Stability of insulin-like growth factor binding protein 2 and matrix metalloproteinase 9. A: Standard curves for insulin-like growth factor binding protein 2 (IGFBP2) and matrix metalloproteinase 9 (MMP9) over an 18 mo period indicates that the assays were stable over time; B: IGFBP2 and MMP9 stability after storage for 18 mo in liquid nitrogen and at -80 ℃ (n = 10 patients); C: Stability of IGFBP2 and MMP9 following three freeze/thaw cycles. All data are represented as average ± standard deviation. F/T: Freeze/thaw cycles. [a]$P < 0.05$ when compared to samples measured immediately.

dent cohorts. Also of importance are age/gender balance, and an accurate representation and understanding of what comprises the normal or control population for the disease being studied. It is recognised that cohort selection, in both control and disease cohorts, is a potential source of bias that can invalidate results of biomarker studies[52,62-64]. In many cases the choice of the control population is obvious. For example, to investigate bio-

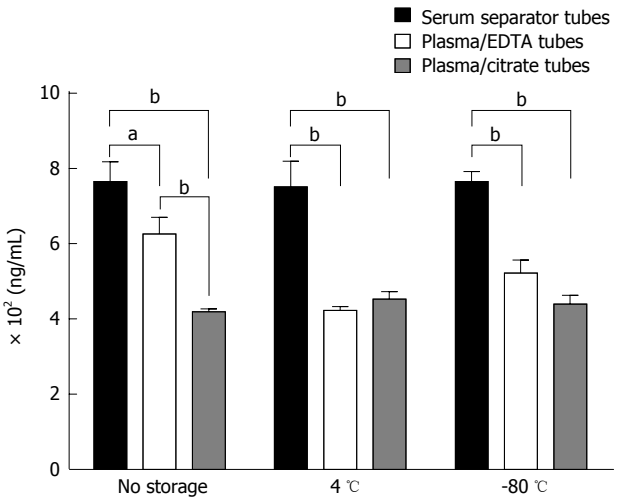


Figure 2 Comparison of insulin-like growth factor binding protein 2 measurements after collection into serum separator tubes, plasma/EDTA tubes and plasma/citrate tubes. Data are represented as average ± SE of the mean for triplicate measurements. $^{a}P < 0.05$; $^{b}P < 0.01$.

markers for breast cancer, the control cohort should be predominantly female, for prostate cancer the control cohort should be male, and when studying childhood diseases, the control cohort should consist of children of the appropriate age range. However, Ransohoff and Gourlay[64] have highlighted numerous examples in the literature where inappropriate selection of patients in the control cohort resulted in identification of biomarkers that were incorrectly associated with the disease conditions. In our own studies on CRC biomarkers, our target control population consists of males and females over the age of 50 years with no previous history of cancer. Additionally, we are also aiming to recruit a control cohort of people that have undergone colonoscopy and who do not have adenomas or colorectal polyps. This is consistent with the clinical distribution of sporadic disease where men and women > 50 years of age represent approximately 80% of all CRC diagnoses[5]. However, this group of aging patients will frequently be taking a number of medications and may have other underlying medical conditions. Indeed, it could be argued that a better control group would be younger patients where CRC itself is uncommon. Longitudinal studies, which involves repeated observations on the same person over long periods of time have been proposed as a better approach as they eliminate confounding invariant personal factors which may be found in cross-sectional studies[4,65]. The Aspirin in Reducing Events in the Elderly study, in which samples from 19000 healthy participants, both males and females, aged 65 years or older are being collected and followed for an average of 5 years may provide an invaluable resource for such studies[66].

An example of difficulties in assigning the correct control levels is shown in Figure 3, where variations between data for IGFBP2 levels in two independent control cohorts that were recruited from different sources was apparent. Cohort 1 (n = 52) consisted of two groups:

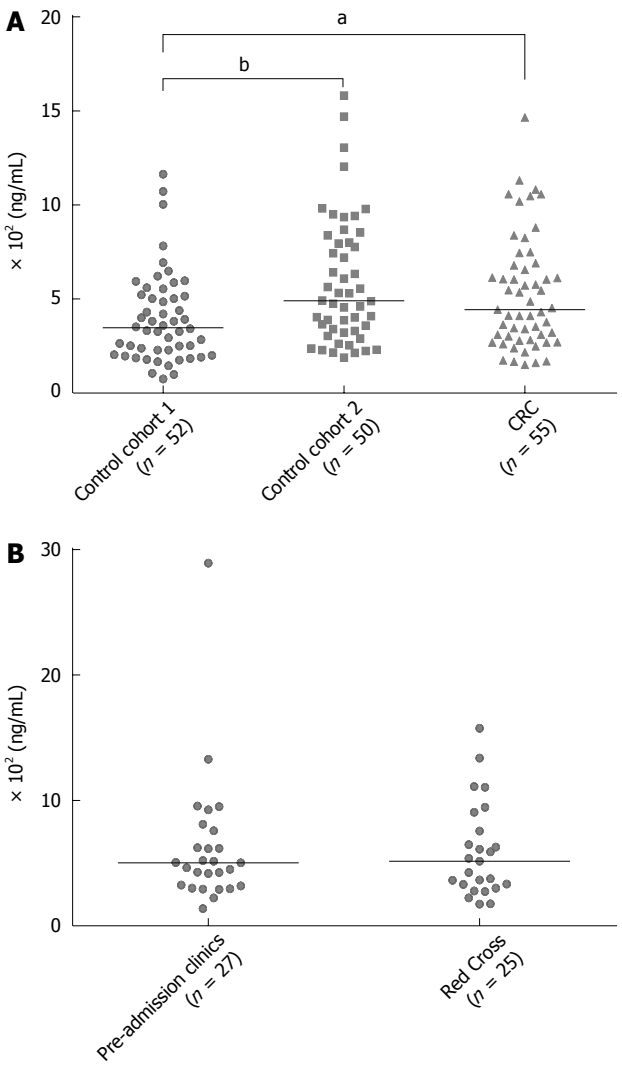


Figure 3 Insulin-like growth factor binding protein 2 measured in different control cohorts and compared to the colorectal cancer patient group. A: Insulin-like growth factor binding protein 2 (IGFBP2) levels in the sera of patients from two different control cohorts and in a colorectal cancer cohort; B: IGFBP2 levels in the sera of control patients recruited from pre-admission clinics (n = 27) and the Red Cross Blood Donation Centre (n = 25). CRC: Colorectal cancer. $^{a}P < 0.05$, $^{b}P < 0.01$ between the median values.

staff, relatives and visitors of patients attending pre-admission clinical centres (n = 40) and patients who were diagnosed with minor medical conditions (orthopaedic clinic or vascular clinic, n = 12) and who did not have a previous history of gastrointestinal disease or cancer. For cohort 2 (n = 50), volunteers were blood donors recruited from Red Cross Blood Donation Centres. Each cohort was balanced for age and gender. Although both cohorts could be considered as representative of the normal population, the median IGFBP2 concentration and the concentration range differed significantly between these two control cohorts (348 ng/mL *vs* 491 ng/mL in cohort 1 and cohort 2, respectively, $P < 0.002$). This difference could not be ascribed to the 12 patients with medical conditions ($P > 0.05$ between patients and staff/visitors) and this result was not reproduced in a smaller independent study we conducted comparing volunteers

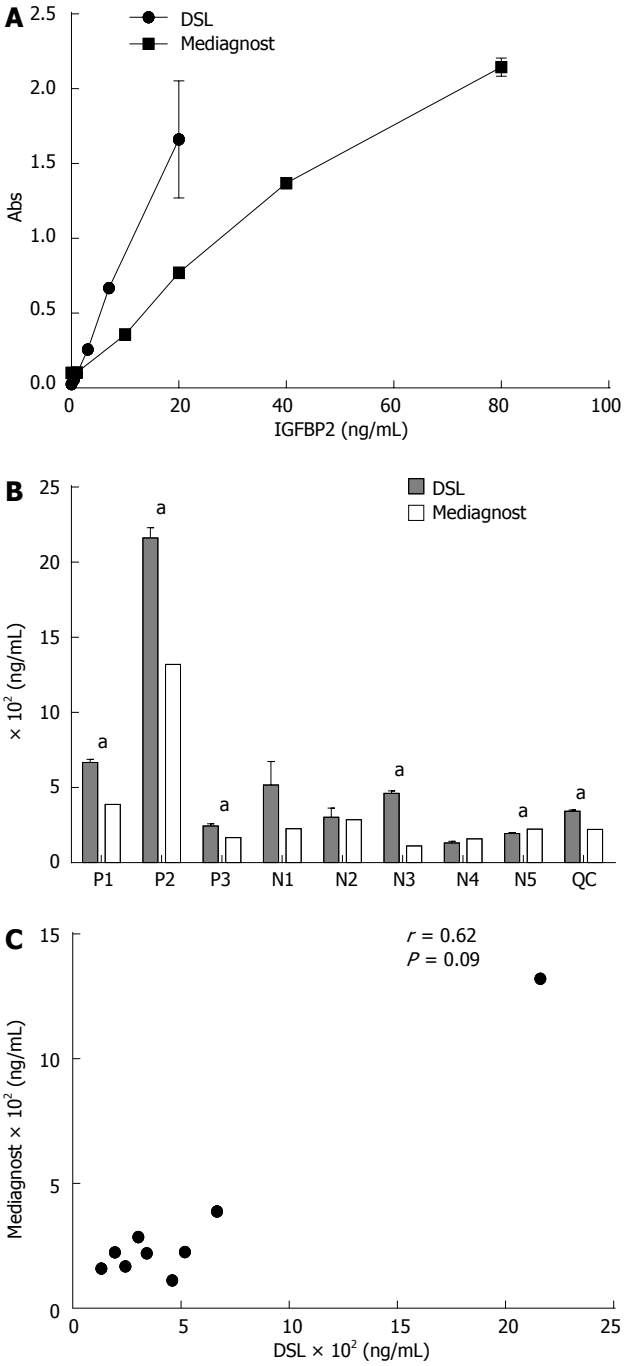


Figure 4 Insulin-like growth factor binding protein 2 measured in patient sera using enzyme-linked immunosorbent assay kits sourced from two different manufacturers. A: Standard curves for insulin-like growth factor binding protein 2 (IGFBP2) enzyme-linked immunosorbent assays sourced from DSL Inc (Texas, United States) and Mediagnost (Kiel, Germany); B: Comparison of IGFBP2 levels in three colorectal cancer patients (P1-P3), five control patients (N1-N5) and a quality control sample consisting of 10 pooled samples; C: Correlation of measured values of IGFBP2 between the two different manufacturers. The correlation coefficient was 0.62 (Spearman correlation, $P > 0.05$). Data are represented as average ± standard deviation of three replicate measurements. [a]$P < 0.05$.

recruited from the Red Cross Blood Donation Centres ($n = 25$) and patients from pre-admission clinical centres ($n = 27$, $P > 0.05$, Figure 3B). Furthermore, when we compared cohort 1 and 2 with the CRC group ($n = 55$), a significant difference in IGFBP2 expression was found with cohort 1 only ($P < 0.05$).

Factors such as time of day sampling (*i.e.*, diurnal variation), fasting *vs* non-fasting states, comorbities, medications, supplements, hormones, sampling methods and storage have also been identified as factors that can potentially affect biomarker concentrations. For example, there is evidence to indicate that IGFBP2 levels are not likely to be affected by fasting[67], but might be affected by diet[68] and may be a marker for metabolic syndrome[69]. Additionally, in a study investigating biomarkers for ovarian cancer, Thorpe *et al*[70] identified that prolactin levels were significantly affected by blood collection procedures where levels were elevated in patients who had blood collected at time of surgery *vs* those who did not (*i.e.*, collected up to 39 d prior to surgery). After adjusting for the collection procedure, they determined that any difference in prolactin levels could be attributed entirely to blood sampling processes and not to malignancy. Similarly, Lomholt *et al*[71] identified that the temperature at which samples were handled and cellular contamination of plasma samples influenced TIMP1 levels in plasma. Although we were not able to definitively determine the source of variation in our control cohort, our data highlights the importance of using multiple control groups to identify possible factors that can affect biomarker measurements leading to potential erroneous results.

ANALYTICAL VARIABLES ASSOCIATED WITH COMMERCIALLY AVAILABLE REAGENTS

For our initial analyses, commercially available ELISA kits were sourced, and where possible, identical batch lots from the same manufacturer were used. Figure 4 demonstrates a potential problem associated with reliance on commercial kits for long term studies. ELISA kits for IGFBP2 were purchased from DSL Inc. until the manufacturer discontinued supply. Accordingly, kits were sourced from an alternate vendor (Mediagnost). To determine the potential impact of a change in supplier on the reproducibility of our preliminary results, we conducted a small study comparing the results obtained from identical patient samples ($n = 8$) and a quality control sample that was included in each assay (QC) using the two alternative kits (Figure 4). The QC sample consisted of pooled normal samples ($n = 10$). For six of these patient samples, there was a significant difference (DSL Inc. *vs* Mediagnost, $P < 0.05$) in the measurements obtained using the two different kits (Figure 4B). Although the correlation between the measured values reached 0.62, this was not significant (Spearman correlation, $P > 0.05$; Figure 4C). It should be noted, however, that the sample size was small ($n = 9$).

Studies by Basuyau *et al*[72], Hauffa *et al*[73], and Rymer *et al*[74] have demonstrated the potential impact of technical problems, such as that described above for IGFBP2,

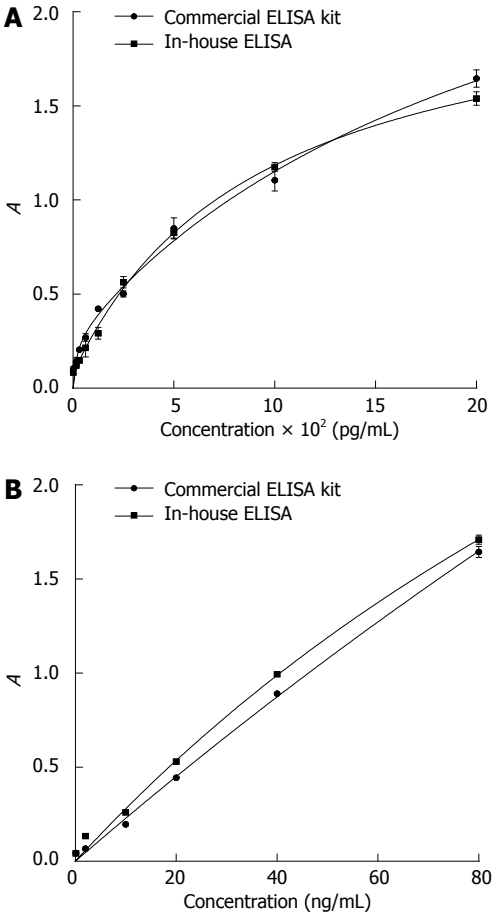


Figure 5 Comparison of calibration curves between commercially available enzyme-linked immunosorbent assay kits and reagents developed in-house for two protein biomarkers. Data are represented as average ± SE of the mean for two replicate measurements.

on the clinical utility of biomarkers. These authors highlight how discrepancies in the values obtained for clinical measurement of prostate specific antigen, CEA, and IGF1/IGFBP3 using different immunoassay methodologies can lead to misdiagnosis of patients[73,74]. Although the source(s) of the discrepancies could not be definitively determined, differences in calibration curves, calibrator standard used ("gold standard") and antibody immunoreactivity were highlighted as potential causes. To understand the impact of changes in methodology, Basuyau *et al*[72] recommended that "known" patient samples be re-evaluated and Hauffa *et al*[73] discussed the importance of a common and well characterised "gold standard" for assay calibration between diagnostic laboratories.

To overcome technical variation due to unforeseen problems such as reproducibility of results between commercially available kits, we have established a pipeline to generate reagents (recombinant protein antigens and renewable high-affinity monoclonal antibodies[75]) for use in multiplexed sandwich ELISA assays for panels of biomarkers that appear to be promising in the initial preliminary phases of our studies. Due to the heterogeneous nature of CRC, and a significant overlap of cancer with other non-malignant pathologies, it is now recognized that the paradigm of a single biomarker to detect an individual cancer may not be realistic, and that panels of biomarkers, which reflect different aspects of the cancer biology, will be required[43,76]. Multiplexed analyses (*e.g.*, Luminex, www.luminex.com) offer significant advantages in terms of overall assay time, reagent costs and, most importantly, reduced sample requirements[77]. To generate panels of monoclonal antibodies in mice or rats for ELISA development, soluble proteins are expressed in mammalian host cell lines to ensure the corresponding post-translational modifications found in endogenous proteins are present. The recombinant target proteins are rigorously analysed using tools such as mass spectrometry and amino acid analysis for protein sequence verification. The monoclonal antibodies generated are validated by ELISA, microarray Western blotting and surface plasmon resonance based technology [*e.g.*, Biacore (www.biacore.com), Proteon (www.bio-rad.com)] for antibody/antigen selectivity, binding kinetics and epitope binding. Once established, the immunoassays are compared and assessed against the commercial kits that were used as part of the original analysis. Figure 5 shows the comparison between the calibration curves derived from commercial kits and from our own reagents for two of our markers. It can be seen that, in both cases, the assay sensitivity and standard curves generated are similar.

CONCLUSION

The identification of panels of sensitive and specific blood-based protein markers for the early diagnosis and surveillance of CRC is recognised as being fundamental to improve survival for this disease. It is widely accepted that a panel of biomarkers that reflects the heterogeneity of the disease will be more successful at diagnosing CRC than a single biomarker. This is supported by the inability of the currently tested biomarkers to diagnose CRC with the sensitivity and specificity required for routine clinical use. Studies involving analyses of multiple biomarkers, such as that undertaken by us and other research groups worldwide, require consideration of many potential confounding issues, some of which unfortunately are impossible or difficult to control for. Of equal importance, and not discussed here, is the need for robust statistical analysis of the data. Implementation of strategies which can overcome and account for potentially confounding variables is essential to ensure robust verification and validation of potential biomarkers and their successful evaluation in large and meaningful clinical cohorts that are representative of the target population, ultimately with successful translation into the clinic.

ACKNOWLEDGMENTS

The authors would like to thank the Victorian Cancer Biobank and the Australian Red Cross for collection of samples.

REFERENCES

1 **Ferlay J**, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. GLOBOCAN 2008 v1.2, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]. Lyon, France: International Agency for Research on Cancer, 2010

2 **Yabroff KR**, Lund J, Kepka D, Mariotto A. Economic burden of cancer in the United States: estimates, projections, and future research. *Cancer Epidemiol Biomarkers Prev* 2011; **20**: 2006-2014 [PMID: 21980008 DOI: 10.1158/1055-9965.epi-11-0650]

3 Food, nutrition, physical activity, and the prevention of cancer: a global perspective. Washington, DC: AICR, 2007

4 **Etzioni R**, Urban N, Ramsey S, McIntosh M, Schwartz S, Reid B, Radich J, Anderson G, Hartwell L. The case for early detection. *Nat Rev Cancer* 2003; **3**: 243-252 [PMID: 12671663 DOI: 10.1038/nrc1041]

5 **Kuipers EJ**, Rösch T, Bretthauer M. Colorectal cancer screening--optimizing current strategies and new directions. *Nat Rev Clin Oncol* 2013; **10**: 130-142 [PMID: 23381005 DOI: 10.1038/nrclinonc.2013.12]

6 **Allison JE**, Tekawa IS, Ransom LJ, Adrain AL. A comparison of fecal occult-blood tests for colorectal-cancer screening. *N Engl J Med* 1996; **334**: 155-159 [PMID: 8531970 DOI: 10.1056/nejm199601183340304]

7 **Morikawa T**, Kato J, Yamaji Y, Wada R, Mitsushima T, Shiratori Y. A comparison of the immunochemical fecal occult blood test and total colonoscopy in the asymptomatic population. *Gastroenterology* 2005; **129**: 422-428 [PMID: 16083699 DOI: 10.1016/j.gastro.2005.05.056]

8 **Parra-Blanco A**, Gimeno-García AZ, Quintero E, Nicolás D, Moreno SG, Jiménez A, Hernández-Guerra M, Carrillo-Palau M, Eishi Y, López-Bastida J. Diagnostic accuracy of immunochemical versus guaiac faecal occult blood tests for colorectal cancer screening. *J Gastroenterol* 2010; **45**: 703-712 [PMID: 20157748 DOI: 10.1007/s00535-010-0214-8]

9 **Wagner PD**, Srivastava S. New paradigms in translational science research in cancer biomarkers. *Transl Res* 2012; **159**: 343-353 [PMID: 22424436 DOI: 10.1016/j.trsl.2012.01.015]

10 **Agesen TH**, Sveen A, Merok MA, Lind GE, Nesbakken A, Skotheim RI, Lothe RA. ColoGuideEx: a robust gene classifier specific for stage II colorectal cancer prognosis. *Gut* 2012; **61**: 1560-1567 [PMID: 22213796 DOI: 10.1136/gutjnl-2011-301179]

11 **Han M**, Liew CT, Zhang HW, Chao S, Zheng R, Yip KT, Song ZY, Li HM, Geng XP, Zhu LX, Lin JJ, Marshall KW, Liew CC. Novel blood-based, five-gene biomarker set for the detection of colorectal cancer. *Clin Cancer Res* 2008; **14**: 455-460 [PMID: 18203981 DOI: 10.1158/1078-0432.ccr-07-1801]

12 **Maak M**, Simon I, Nitsche U, Roepman P, Snel M, Glas AM, Schuster T, Keller G, Zeestraten E, Goossens I, Janssen KP, Friess H, Rosenberg R. Independent validation of a prognostic genomic signature (ColoPrint) for patients with stage II colon cancer. *Ann Surg* 2013; **257**: 1053-1058 [PMID: 23295318 DOI: 10.1097/SLA.0b013e31827c1180]

13 **Marshall KW**, Mohr S, Khettabi FE, Nossova N, Chao S, Bao W, Ma J, Li XJ, Liew CC. A blood-based biomarker panel for stratifying current risk for colorectal cancer. *Int J Cancer* 2010; **126**: 1177-1186 [PMID: 19795455 DOI: 10.1002/ijc.24910]

14 **Salazar R**, Roepman P, Capella G, Moreno V, Simon I, Dreezen C, Lopez-Doriga A, Santos C, Marijnen C, Westerga J, Bruin S, Kerr D, Kuppen P, van de Velde C, Morreau H, Van Velthuysen L, Glas AM, Van't Veer LJ, Tollenaar R. Gene expression signature to improve prognosis prediction of stage II and III colorectal cancer. *J Clin Oncol* 2011; **29**: 17-24 [PMID: 21098318 DOI: 10.1200/jco.2010.30.1077]

15 **Yip KT**, Das PK, Suria D, Lim CR, Ng GH, Liew CC. A case-controlled validation study of a blood-based seven-gene biomarker panel for colorectal cancer in Malaysia. *J Exp Clin Cancer Res* 2010; **29**: 128 [PMID: 20846378 DOI: 10.1186/1756-9966-29-128]

16 **Bünger S**, Haug U, Kelly FM, Klempt-Giessing K, Cartwright A, Posorski N, Dibbelt L, Fitzgerald SP, Bruch HP, Roblick UJ, von Eggeling F, Brenner H, Habermann JK. Toward standardized high-throughput serum diagnostics: multiplex-protein array identifies IL-8 and VEGF as serum markers for colon cancer. *J Biomol Screen* 2011; **16**: 1018-1026 [PMID: 21807963 DOI: 10.1177/1087057111414894]

17 **Shimwell NJ**, Wei W, Wilson S, Wakelam MJ, Ismail T, Iqbal T, Johnson PJ, Martin A, Ward DG. Assessment of novel combinations of biomarkers for the detection of colorectal cancer. *Cancer Biomark* 2010; **7**: 123-132 [PMID: 21263188 DOI: 10.3233/cbm-2010-0155]

18 **Tao S**, Haug U, Kuhn K, Brenner H. Comparison and combination of blood-based inflammatory markers with faecal occult blood tests for non-invasive colorectal cancer screening. *Br J Cancer* 2012; **106**: 1424-1430 [PMID: 22454079 DOI: 10.1038/bjc.2012.104]

19 **Wild N**, Andres H, Rollinger W, Krause F, Dilba P, Tacke M, Karl J. A combination of serum markers for the early detection of colorectal cancer. *Clin Cancer Res* 2010; **16**: 6111-6121 [PMID: 20798228 DOI: 10.1158/1078-0432.ccr-10-0119]

20 **Ritchie SA**, Ahiahonu PW, Jayasinghe D, Heath D, Liu J, Lu Y, Jin W, Kavianpour A, Yamazaki Y, Khan AM, Hossain M, Su-Myat KK, Wood PL, Krenitsky K, Takemasa I, Miyake M, Sekimoto M, Monden M, Matsubara H, Nomura F, Goodenowe DB. Reduced levels of hydroxylated, polyunsaturated ultra long-chain fatty acids in the serum of colorectal cancer patients: implications for early screening and detection. *BMC Med* 2010; **8**: 13 [PMID: 20156336 DOI: 10.1186/1741-7015-8-13]

21 **Ahlquist DA**, Zou H, Domanico M, Mahoney DW, Yab TC, Taylor WR, Butz ML, Thibodeau SN, Rabeneck L, Paszat LF, Kinzler KW, Vogelstein B, Bjerregaard NC, Laurberg S, Sørensen HT, Berger BM, Lidgard GP. Next-generation stool DNA test accurately detects colorectal cancer and large adenomas. *Gastroenterology* 2012; **142**: 248-56; quiz e25-6 [PMID: 22062357 DOI: 10.1053/j.gastro.2011.10.031]

22 **Grützmann R**, Molnar B, Pilarsky C, Habermann JK, Schlag PM, Saeger HD, Miehlke S, Stolz T, Model F, Roblick UJ, Bruch HP, Koch R, Liebenberg V, Devos T, Song X, Day RH, Sledziewski AZ, Lofton-Day C. Sensitive detection of colorectal cancer in peripheral blood by septin 9 DNA methylation assay. *PLoS One* 2008; **3**: e3759 [PMID: 19018278 DOI: 10.1371/journal.pone.0003759]

23 **deVos T**, Tetzner R, Model F, Weiss G, Schuster M, Distler J, Steiger KV, Grützmann R, Pilarsky C, Habermann JK, Fleshner PR, Oubre BM, Day R, Sledziewski AZ, Lofton-Day C. Circulating methylated SEPT9 DNA in plasma is a biomarker for colorectal cancer. *Clin Chem* 2009; **55**: 1337-1346 [PMID: 19406918 DOI: 10.1373/clinchem.2008.115808]

24 **Tóth K**, Sipos F, Kalmár A, Patai AV, Wichmann B, Stoehr R, Golcher H, Schellerer V, Tulassay Z, Molnár B. Detection of methylated SEPT9 in plasma is a reliable screening method for both left- and right-sided colon cancers. *PLoS One* 2012; **7**: e46000 [PMID: 23049919 DOI: 10.1371/journal.pone.0046000]

25 **Warren JD**, Xiong W, Bunker AM, Vaughn CP, Furtado LV, Roberts WL, Fang JC, Samowitz WS, Heichman KA. Septin 9 methylated DNA is a sensitive and specific blood test for colorectal cancer. *BMC Med* 2011; **9**: 133 [PMID: 22168215 DOI: 10.1186/1741-7015-9-133]

26 **Church TR**, Wandell M, Lofton-Day C, Mongin SJ, Burger M, Payne SR, Castaños-Vélez E, Blumenstein BA, Rösch T, Osborn N, Snover D, Day RW, Ransohoff DF. Prospective evaluation of methylated SEPT9 in plasma for detection of asymptomatic colorectal cancer. *Gut* 2014; **63**: 317-325 [PMID: 23408352 DOI: 10.1136/gutjnl-2012-304149]

27 **Ahlquist DA**, Taylor WR, Mahoney DW, Zou H, Domanico M, Thibodeau SN, Boardman LA, Berger BM, Lidgard GP.

The stool DNA test is more accurate than the plasma septin 9 test in detecting colorectal neoplasia. *Clin Gastroenterol Hepatol* 2012; **10**: 272-7.e1 [PMID: 22019796 DOI: 10.1016/j.cgh.2011.10.008]

28 **Ladabaum U**, Allen J, Wandell M, Ramsey S. Colorectal cancer screening with blood-based biomarkers: cost-effectiveness of methylated septin 9 DNA versus current strategies. *Cancer Epidemiol Biomarkers Prev* 2013; **22**: 1567-1576 [PMID: 23796793 DOI: 10.1158/1055-9965.epi-13-0204]

29 **Hardt PD**, Ngoumou BK, Rupp J, Schnell-Kretschmer H, Kloer HU. Tumor M2-pyruvate kinase: a promising tumor marker in the diagnosis of gastro-intestinal cancer. *Anticancer Res* 2000; **20**: 4965-4968 [PMID: 11326648]

30 **Hardt PD**, Toepler M, Ngoumou B, Rupp J, Kloer HU. Measurement of fecal pyruvate kinase type M2 (tumor M2-PK) concentrations in patients with gastric cancer, colorectal cancer, colorectal adenomas and controls. *Anticancer Res* 2003; **23**: 851-853 [PMID: 12820312]

31 **Koss K**, Maxton D, Jankowski JA. Faecal dimeric M2 pyruvate kinase in colorectal cancer and polyps correlates with tumour staging and surgical intervention. *Colorectal Dis* 2008; **10**: 244-248 [PMID: 17784868 DOI: 10.1111/j.1463-1318.2007.01334.x]

32 **Nielsen HJ**, Brünner N, Frederiksen C, Lomholt AF, King D, Jørgensen LN, Olsen J, Rahr HB, Thygesen K, Hoyer U, Laurberg S, Christensen IJ. Plasma tissue inhibitor of metalloproteinases-1 (TIMP-1): a novel biological marker in the detection of primary colorectal cancer. Protocol outlines of the Danish-Australian endoscopy study group on colorectal cancer detection. *Scand J Gastroenterol* 2008; **43**: 242-248 [PMID: 18224568 DOI: 10.1080/00365520701523439]

33 **Nielsen HJ**, Brünner N, Jorgensen LN, Olsen J, Rahr HB, Thygesen K, Hoyer U, Laurberg S, Stieber P, Blankenstein MA, Davis G, Dowell BL, Christensen IJ. Plasma TIMP-1 and CEA in detection of primary colorectal cancer: a prospective, population based study of 4509 high-risk individuals. *Scand J Gastroenterol* 2011; **46**: 60-69 [PMID: 20799911 DOI: 10.3109/00365521.2010.513060]

34 **Holten-Andersen MN**, Christensen IJ, Nielsen HJ, Stephens RW, Jensen V, Nielsen OH, Sørensen S, Overgaard J, Lilja H, Harris A, Murphy G, Brünner N. Total levels of tissue inhibitor of metalloproteinases 1 in plasma yield high diagnostic sensitivity and specificity in patients with colon cancer. *Clin Cancer Res* 2002; **8**: 156-164 [PMID: 11801553]

35 **Holten-Andersen MN**, Fenger C, Nielsen HJ, Rasmussen AS, Christensen IJ, Brünner N, Kronborg O. Plasma TIMP-1 in patients with colorectal adenomas: a prospective study. *Eur J Cancer* 2004; **40**: 2159-2164 [PMID: 15341992 DOI: 10.1016/j.ejca.2004.06.011]

36 **Holten-Andersen MN**, Stephens RW, Nielsen HJ, Murphy G, Christensen IJ, Stetler-Stevenson W, Brünner N. High preoperative plasma tissue inhibitor of metalloproteinase-1 levels are associated with short survival of patients with colorectal cancer. *Clin Cancer Res* 2000; **6**: 4292-4299 [PMID: 11106246]

37 **Ishida H**, Murata N, Hayashi Y, Tada M, Hashimoto D. Serum levels of tissue inhibitor of metalloproteinases-1 (TIMP-1) in colorectal cancer patients. *Surg Today* 2003; **33**: 885-892 [PMID: 14669077 DOI: 10.1007/s00595-003-2628-x]

38 **Pellegrini P**, Contasta I, Berghella AM, Gargano E, Mammarella C, Adorno D. Simultaneous measurement of soluble carcinoembryonic antigen and the tissue inhibitor of metalloproteinase TIMP1 serum levels for use as markers of preinvasive to invasive colorectal cancer. *Cancer Immunol Immunother* 2000; **49**: 388-394 [PMID: 10999465]

39 **Waas ET**, Wobbes T, Ruers T, Lomme RM, Hendriks T. Circulating gelatinases and tissue inhibitor of metalloproteinase-1 in colorectal cancer metastatic liver disease. *Eur J Surg Oncol* 2006; **32**: 756-763 [PMID: 16650961 DOI: 10.1016/j.ejso.2006.03.028]

40 **Mulder SA**, van Leerdam ME, van Vuuren AJ, Francke J, van Toorenenbergen AW, Kuipers EJ, Ouwendijk RJ. Tumor pyruvate kinase isoenzyme type M2 and immunochemical fecal occult blood test: performance in screening for colorectal cancer. *Eur J Gastroenterol Hepatol* 2007; **19**: 878-882 [PMID: 17873612 DOI: 10.1097/MEG.0b013e3282cfa49c]

41 **Hundt S**, Haug U, Brenner H. Blood markers for early detection of colorectal cancer: a systematic review. *Cancer Epidemiol Biomarkers Prev* 2007; **16**: 1935-1953 [PMID: 17932341 DOI: 10.1158/1055-9965.epi-06-0994]

42 **Kim HJ**, Yu MH, Kim H, Byun J, Lee C. Noninvasive molecular biomarkers for the detection of colorectal cancer. *BMB Rep* 2008; **41**: 685-692 [PMID: 18959813]

43 **Duffy MJ**, van Dalen A, Haglund C, Hansson L, Klapdor R, Lamerz R, Nilsson O, Sturgeon C, Topolcan O. Clinical utility of biochemical markers in colorectal cancer: European Group on Tumour Markers (EGTM) guidelines. *Eur J Cancer* 2003; **39**: 718-727 [PMID: 12651195]

44 **Wang WS**, Lin JK, Chiou TJ, Liu JH, Fan FS, Yen CC, Lin TC, Jiang JK, Yang SH, Wang HS, Chen PM. Preoperative carcinoembryonic antigen level as an independent prognostic factor in colorectal cancer: Taiwan experience. *Jpn J Clin Oncol* 2000; **30**: 12-16 [PMID: 10770562]

45 **Galli C**, Basso D, Plebani M. CA 19-9: handle with care. *Clin Chem Lab Med* 2013; **51**: 1369-1383 [PMID: 23370912 DOI: 10.1515/cclm-2012-0744]

46 **Reiter W**, Stieber P, Reuter C, Nagel D, Lau-Werner U, Lamerz R. Multivariate analysis of the prognostic value of CEA and CA 19-9 serum levels in colorectal cancer. *Anticancer Res* 2000; **20**: 5195-5198 [PMID: 11326694]

47 **Wang JY**, Lu CY, Chu KS, Ma CJ, Wu DC, Tsai HL, Yu FJ, Hsieh JS. Prognostic significance of pre- and postoperative serum carcinoembryonic antigen levels in patients with colorectal cancer. *Eur Surg Res* 2007; **39**: 245-250 [PMID: 17457032 DOI: 10.1159/000101952]

48 **Glas AM**, Floore A, Delahaye LJ, Witteveen AT, Pover RC, Bakx N, Lahti-Domenici JS, Bruinsma TJ, Warmoes MO, Bernards R, Wessels LF, Van't Veer LJ. Converting a breast cancer microarray signature into a high-throughput diagnostic test. *BMC Genomics* 2006; **7**: 278 [PMID: 17074082 DOI: 10.1186/1471-2164-7-278]

49 **Ross JS**, Hatzis C, Symmans WF, Pusztai L, Hortobágyi GN. Commercialized multigene predictors of clinical outcome for breast cancer. *Oncologist* 2008; **13**: 477-493 [PMID: 18515733 DOI: 10.1634/theoncologist.2007-0248]

50 **O'Connell MJ**, Lavery I, Yothers G, Paik S, Clark-Langone KM, Lopatin M, Watson D, Baehner FL, Shak S, Baker J, Cowens JW, Wolmark N. Relationship between tumor gene expression and recurrence in four independent studies of patients with stage II/III colon cancer treated with surgery alone or surgery plus adjuvant fluorouracil plus leucovorin. *J Clin Oncol* 2010; **28**: 3937-3944 [PMID: 20679606 DOI: 10.1200/jco.2010.28.9538]

51 **Sveen A**, Ågesen TH, Nesbakken A, Meling GI, Rognum TO, Liestøl K, Skotheim RI, Lothe RA. ColoGuidePro: a prognostic 7-gene expression signature for stage III colorectal cancer patients. *Clin Cancer Res* 2012; **18**: 6001-6010 [PMID: 22991413 DOI: 10.1158/1078-0432.ccr-11-3302]

52 **Tao S**, Hundt S, Haug U, Brenner H. Sensitivity estimates of blood-based tests for colorectal cancer detection: impact of overrepresentation of advanced stage disease. *Am J Gastroenterol* 2011; **106**: 242-253 [PMID: 20959816 DOI: 10.1038/ajg.2010.393]

53 **Olson J**, Whitney DH, Durkee K, Shuber AP. DNA stabilization is critical for maximizing performance of fecal DNA-based colorectal cancer tests. *Diagn Mol Pathol* 2005; **14**: 183-191 [PMID: 16106201]

54 **Rai AJ**, Gelfand CA, Haywood BC, Warunek DJ, Yi J, Schuchard MD, Mehigh RJ, Cockrill SL, Scott GB, Tammen H, Schulz-Knappe P, Speicher DW, Vitzthum F, Haab BB, Siest G, Chan DW. HUPO Plasma Proteome Project speci-

men collection and handling: towards the standardization of parameters for plasma proteome samples. *Proteomics* 2005; **5**: 3262-3277 [PMID: 16052621 DOI: 10.1002/pmic.200401245]

55 **Tuck MK**, Chan DW, Chia D, Godwin AK, Grizzle WE, Krueger KE, Rom W, Sanda M, Sorbara L, Stass S, Wang W, Brenner DE. Standard operating procedures for serum and plasma collection: early detection research network consensus statement standard operating procedure integration working group. *J Proteome Res* 2009; **8**: 113-117 [PMID: 19072545 DOI: 10.1021/pr800545q]

56 **Zou H**, Harrington JJ, Klatt KK, Ahlquist DA. A sensitive method to quantify human long DNA in stool: relevance to colorectal cancer screening. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 1115-1119 [PMID: 16775168 DOI: 10.1158/1055-9965.epi-05-0992]

57 **Brierley GV**, Priebe IK, Purins L, Fung KY, Tabor B, Lockett T, Nice E, Gibbs P, Tie J, McMurrick P, Moore J, Ruszkiewicz A, Burgess A, Cosgrove LJ. Serum concentrations of brain-derived neurotrophic factor (BDNF) are decreased in colorectal cancer patients. *Cancer Biomark* 2013; **13**: 67-73 [PMID: 23838134 DOI: 10.3233/cbm-130345]

58 **Fung KY**, Priebe I, Purins L, Tabor B, Brierley GV, Lockett T, Nice E, Gibbs P, Tie J, McMurrick P, Moore J, Ruszkiewicz A, Burgess A, Cosgrove LJ. Performance of serum lipocalin 2 as a diagnostic marker for colorectal cancer. *Cancer Biomark* 2013; **13**: 75-79 [PMID: 23838135 DOI: 10.3233/cbm-130335]

59 **Gast MC**, van Gils CH, Wessels LF, Harris N, Bonfrer JM, Rutgers EJ, Schellens JH, Beijnen JH. Influence of sample storage duration on serum protein profiles assessed by surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF MS). *Clin Chem Lab Med* 2009; **47**: 694-705 [PMID: 19416081 DOI: 10.1515/cclm.2009.151]

60 **Feng Z**, Kagan J, Pepe M, Thornquist M, Ann Rinaudo J, Dahlgren J, Krueger K, Zheng Y, Patriotis C, Huang Y, Sorbara L, Thompson I, Srivastava S. The Early Detection Research Network's Specimen reference sets: paving the way for rapid evaluation of potential biomarkers. *Clin Chem* 2013; **59**: 68-74 [PMID: 23193062 DOI: 10.1373/clinchem.2012.185140]

61 **Hainaut P**, Vozar B, Rinaldi S, Riboli E, Caboux E. The European Prospective Investigation into Cancer and Nutrition biobank. *Methods Mol Biol* 2011; **675**: 179-191 [PMID: 20949388 DOI: 10.1007/978-1-59745-423-0_7]

62 **McLerran D**, Grizzle WE, Feng Z, Bigbee WL, Banez LL, Cazares LH, Chan DW, Diaz J, Izbicka E, Kagan J, Malehorn DE, Malik G, Oelschlager D, Partin A, Randolph T, Rosenzweig N, Srivastava S, Srivastava S, Thompson IM, Thornquist M, Troyer D, Yasui Y, Zhang Z, Zhu L, Semmes OJ. Analytical validation of serum proteomic profiling for diagnosis of prostate cancer: sources of sample bias. *Clin Chem* 2008; **54**: 44-52 [PMID: 17981926 DOI: 10.1373/clinchem.2007.091470]

63 **Ransohoff DF**. Bias as a threat to the validity of cancer molecular-marker research. *Nat Rev Cancer* 2005; **5**: 142-149 [PMID: 15685197 DOI: 10.1038/nrc1550]

64 **Ransohoff DF**, Gourlay ML. Sources of bias in specimens for research about molecular markers for cancer. *J Clin Oncol* 2010; **28**: 698-704 [PMID: 20038718 DOI: 10.1200/jco.2009.25.6065]

65 **Woolcott CG**, Courneya KS, Boyd NF, Yaffe MJ, McTiernan A, Brant R, Jones CA, Stanczyk FZ, Terry T, Cook LS, Wang Q, Friedenreich CM. Longitudinal changes in IGF-I and IGFBP-3, and mammographic density among postmenopausal women. *Cancer Epidemiol Biomarkers Prev* 2013; **22**: 2116-2120 [PMID: 24019394 DOI: 10.1158/1055-9965.epi-13-0401]

66 **Nelson M**, Reid C, Beilin L, Donnan G, Johnston C, Krum H, Storey E, Tonkin A, McNeil J. Rationale for a trial of low-dose aspirin for the primary prevention of major adverse cardiovascular events and vascular dementia in the elderly: Aspirin in Reducing Events in the Elderly (ASPREE). *Drugs Aging* 2003; **20**: 897-903 [PMID: 14565783]

67 **Clemmons DR**, Snyder DK, Busby WH. Variables controlling the secretion of insulin-like growth factor binding protein-2 in normal human subjects. *J Clin Endocrinol Metab* 1991; **73**: 727-733 [PMID: 1716260]

68 **Young NJ**, Metcalfe C, Gunnell D, Rowlands MA, Lane JA, Gilbert R, Avery KN, Davis M, Neal DE, Hamdy FC, Donovan J, Martin RM, Holly JM. A cross-sectional analysis of the association between diet and insulin-like growth factor (IGF)-I, IGF-II, IGF-binding protein (IGFBP)-2, and IGFBP-3 in men in the United Kingdom. *Cancer Causes Control* 2012; **23**: 907-917 [PMID: 22527168 DOI: 10.1007/s10552-012-9961-6]

69 **Heald AH**, Kaushal K, Siddals KW, Rudenski AS, Anderson SG, Gibson JM. Insulin-like growth factor binding protein-2 (IGFBP-2) is a marker for the metabolic syndrome. *Exp Clin Endocrinol Diabetes* 2006; **114**: 371-376 [PMID: 16915540 DOI: 10.1055/s-2006-924320]

70 **Thorpe JD**, Duan X, Forrest R, Lowe K, Brown L, Segal E, Nelson B, Anderson GL, McIntosh M, Urban N. Effects of blood collection conditions on ovarian cancer serum markers. *PLoS One* 2007; **2**: e1281 [PMID: 18060075 DOI: 10.1371/journal.pone.0001281]

71 **Lomholt AF**, Frederiksen CB, Christensen IJ, Brünner N, Nielsen HJ. Plasma tissue inhibitor of metalloproteinases-1 as a biological marker? Pre-analytical considerations. *Clin Chim Acta* 2007; **380**: 128-132 [PMID: 17328880 DOI: 10.1016/j.cca.2007.01.022]

72 **Basuyau JP**, Leroy M, Brunelle P. Determination of tumor markers in serum. Pitfalls and good practice. *Clin Chem Lab Med* 2001; **39**: 1227-1233 [PMID: 11798082 DOI: 10.1515/cclm.2001.197]

73 **Hauffa BP**, Lehmann N, Bettendorf M, Mehls O, Dörr HG, Stahnke N, Steinkamp H, Said E, Ranke MB. Central laboratory reassessment of IGF-I, IGF-binding protein-3, and GH serum concentrations measured at local treatment centers in growth-impaired children: implications for the agreement between outpatient screening and the results of somatotropic axis functional testing. *Eur J Endocrinol* 2007; **157**: 597-603 [PMID: 17984239 DOI: 10.1530/eje-07-0338]

74 **Rymer JC**, Sabatier R, Daver A, Bourleaud J, Assicot M, Bremond J, Rapin J, Salhi SL, Thirion B, Vassault A, Ingrand J, Pau B. A new approach for clinical biological assay comparison and standardization: application of principal component analysis to a multicenter study of twenty-one carcinoembryonic antigen immunoassay kits. *Clin Chem* 1999; **45**: 869-881 [PMID: 10351997]

75 **Colwill K**, Gräslund S. A roadmap to generate renewable protein binders to the human proteome. *Nat Methods* 2011; **8**: 551-558 [PMID: 21572409 DOI: 10.1038/nmeth.1607]

76 **Baker M**. In biomarkers we trust? *Nat Biotechnol* 2005; **23**: 297-304 [PMID: 15765081 DOI: 10.1038/nbt0305-297]

77 **Fu Q**, Schoenhoff FS, Savage WJ, Zhang P, Van Eyk JE. Multiplex assays for biomarker research and clinical application: translational science coming of age. *Proteomics Clin Appl* 2010; **4**: 271-284 [PMID: 21137048 DOI: 10.1002/prca.200900217]

P- Reviewers: Langner C, Tanase CP **S- Editor**: Gou SX
L- Editor: A **E- Editor**: Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.899

World J Gastroenterol 2014 January 28; 20(4): 899-907
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

How to select the optimal treatment for first line metastatic colorectal cancer

Alexander Stein, Carsten Bokemeyer

Alexander Stein, Carsten Bokemeyer, Department of Oncology, Haematology, Stem Cell Transplantation with the Section Pneumology, Hubertus Wald Tumor Centre University Cancer Centre Hamburg, University Medical Centre Hamburg-Eppendorf, 20246 Hamburg, Germany

Author contributions: Both authors contributed equally to this work, designed and performed the research, analyzed the data, and wrote the paper.

Correspondence to: Alexander Stein, MD, Department of Oncology, Haematology, Stem Cell Transplantation with the Section Pneumology, Hubertus Wald Tumor Centre University Cancer Centre Hamburg, University Medical Centre Hamburg-Eppendorf, Martinistr. 52, 20246 Hamburg, Germany. a.stein@uke.de

Telephone: +49-40-741056882 Fax: +49-40-741056744
Received: September 25, 2013 Revised: October 27, 2013
Accepted: December 12, 2013
Published online: January 28, 2014

Abstract

Choice of first line treatment for patients with metastatic colorectal cancer (mCRC) is based on tumour and patient related factors and molecular information for determination of individual treatment aim and thus treatment intensity. Recent advances (*e.g.*, extended *RAS* testing) enable tailored patient assignment to the most beneficial treatment approach. Besides fluoropyrimidines, irinotecan and oxaliplatin, a broad variety of molecular targeting agents are currently available, *e.g.*, anti-angiogenic agents (bevacizumab) and epidermal growth factor receptor (EGFR) antibodies (cetuximab, panitumumab) for first line treatment of mCRC. Although some combinations should be avoided (*e.g.*, oral or bolus fluoropyrimidines, oxaliplatin and EGFR antibodies), treatment options range from single agent to highly effective four-drug regimen. Preliminary data comparing EGFR antibodies and bevacizumab, both with chemotherapy, seem to favour EGFR antibodies in *RAS* wildtype disease. However, choosing the most appropriate treatment approach for mCRC patients remains a complex issue, with numerous open questions.

Key words: Colorectal cancer; Metastatic; Induction chemotherapy; Epidermal growth factor receptor

Core tip: Selection of the optimal first line treatment for metastatic colorectal cancer is a complex issue influencing course of disease and most likely survival of the individual patient. Available data will be analyzed to allow for a patient and disease specific, molecularly stratified treatment approach, applying systemic treatment (chemotherapy and antibodies) and locally ablative measures (surgery and radiofrequency ablation).

Stein A, Bokemeyer C. How to select the optimal treatment for first line metastatic colorectal cancer. *World J Gastroenterol* 2014; 20(4): 899-907 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/899.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.899

INTRODUCTION

After lung (1.61 million cases) and breast cancer (1.38 million), colorectal cancer (CRC, 1.23 million) is one of the most commonly diagnosed malignancies worldwide[1]. Moreover, after lung cancer, CRC is the second most common cause of cancer deaths[2]. Around one quarter of patients with CRC present with metastatic disease at time of diagnosis (synchronous disease), and up to 40% of patients will develop metastases during the course of their disease, resulting in a relatively high overall mortality rate associated with CRC.

As a result of recent advances in the treatment of

Table 1 Prognostic scores/health assessments

Score	Risk category	Factors
"Kohne" score[13]	Low risk	ECOG 0/1 and only one tumour site
	Intermediate risk	ECOG 0/1, ALP < 300 U/L and more than one tumour site or ECOG > 1 and WBC < 1 × 10^{10}/L and only one tumour site
	High risk	ECOG 0/1 and more than one tumour site and ALP ⩾ 300 U/L or ECOG > 1 and more than one tumour site or ECOG > 1 and WBC > 1 × 10^{10}/L
FOCUS 2[15]	Comprehensive health assessment at baseline limited health	Weight change Timed 20 metre walk MMSE CCI
	Assessment during course of treatment (excluding MMSE and CCI)	Patient completed questionnaire (social activity, physical fitness, symptoms, overall quality of life and depression)

ECOG: Eastern collaborative oncology group performance status; ALP: Alkaline phosphatase; WBC: White blood cells; MMSE: Mini mental state examination; CCI: Charlson comorbidity index.

metastatic colorectal cancer (mCRC), median overall survival (OS) can now be as long as 30 mo in selected patient groups and up to 70% of patients will receive at least two lines of treatment[3-7]. Several drugs as single agent or in various combinations are available for mCRC, including fluoropyrimidines (5FU, capecitabine), irinotecan, oxaliplatin, the vascular endothelial growth factor (VEGF) antibody bevacizumab, the epidermal growth factor receptor (EGFR) antibodies cetuximab and panitumumab for *RAS* wildtype patients, the VEGF receptors 1 and 2 fusion protein aflibercept and the multitarget tyrosine kinase inhibitor regorafenib. Moreover, secondary resection and/or ablation *e.g.*, by surgery or radiofrequency may contribute to long-term survival and even cure, or at least allow a relevant chemotherapy free interval[8,9].

According to recent data, choice of first line treatment seems to be relevant for further course of disease, despite available efficacious second, third and if applicable fourth line regimen and the cross over use of all available drugs in later lines. The aim of this article is to review the available data on choice of first line treatment in mCRC. Pertinent data from published trials and reports and abstracts presented at selected oncology association meetings [American Society of Clinical Oncology and European Society for Medical Oncology (ESMO)/European cancer organisation] until September 2013 were reviewed.

PROGNOSTIC FACTORS FOR PATIENT STRATIFICATION

Prognosis of mCRC depends on several patient related (*e.g.*, age, performance status, co-morbidity), tumour related (*e.g.*, spread of disease, growth dynamics, symptoms, localization in particular liver and/or extrahepatic metastases), biochemical (*e.g.*, baseline values of carcinoembryonic antigen, lactate dehydrogenase, platelets, leucocytes, haemoglobin, alkaline phosphatase, albumine) or molecular factors (*e.g.*, *KRAS* or *NRAS* mutations, *BRAF* mutation)[10]. Whereas *BRAF* mutation is associated with shorter survival, prognostic value of *KRAS* mutation is not clarified yet[11,12]. Some factors are combined to scores, which might be useful for stratification of patients within clinical trials and in daily clinical practise (Table 1)[13-15]. Determination of patients' individual prognoses might be useful for choice of treatment, particularly in regard of intensity of systemic treatment and integration of local ablation into the overall therapeutic concept.

Besides the above-mentioned factors prognostic information can be derived from a broad variety of tissue or blood markers, *e.g.*, circulating tumour cells, levels of growth factor receptor-ligands, mutations or amplifications within the relevant signalling pathways or receptors, or epigenetic alterations[16,17]. These prognostic factors might gain relevance in the future, but are currently neither broadly available nor relevant for clinical decisions[10].

PREDICTIVE FACTORS FOR TREATMENT EFFICACY OR TOXICITY

Despite tremendous efforts in searching for predictive markers in mCRC, only *RAS* mutation have been established, precluding treatment with EGFR antibodies. Initially *KRAS* mutations in exon 2 (codon 12 and 13) have been found to be predictive for non-response to cetuximab or panitumumab[18,19]. Although data are conflicting, *KRAS* codon G13D mutation (16% of *KRAS* mutated tumours) seems not to preclude efficacy of cetuximab in patients with *KRAS* mutations[20,21]. However, neither in the COIN trial, combining oxaliplatin with different fluoropyrimidine schedules and cetuximab, nor in the available panitumumab trials *KRAS* G13D mutated tumours seem to derive relevant benefit from anti-EGFR treatment[22-24].

Recently, retrospective analyses of the PRIME study demonstrated the negative predictive value of *KRAS* mutation in exon 3 and 4 and *NRAS* mutations in exon 2,3 and 4 for treatment with 5FU/leucovorin and oxaliplatin (FOLFOX) and panitumumab[25]. In patients with any RAS mutation the addition of panitumumab to FOLFOX had a detrimental effect on progression free survival (PFS) (HR = 1.31; 95%CI: 1.07-1.60) and OS (HR = 1.21; 95%CI: 1.01-1.45). In contrast, median OS was 25.8 mo *vs* 20.2 mo (HR = 0.77; 95%CI: 0.64-0.94, P = 0.009) in the all *RAS* wild-type population in favour of the combination of panitumumab and FOLFOX. Although data from the cetuximab containing trials (CRYSTAL, OPUS) are not yet available, *RAS* mutational status will likely be of similar impact for cetuximab treatment[26].

Despite the strong adverse prognostic effect of *BRAF* mutation (8% of *RAS* wild-type patients), the predictive value for treatment with EGFR antibodies is still unclear, with some analysis indicating a lack of benefit, particularly in advanced treatment situations[24,26,27], whereas data from first line trials (CRYSTAL, PRIME and OPUS) show

Table 2 European Society for Medical Oncology clinical groups for first line treatment stratification[10]

ESMO group	Clinical presentation	Treatment aim	Treatment intensity
0	Clearly R0-resectable liver and/or lung metastases	Decrease risk of or delay relapse	FOLFOX
1	Liver and/or lung metastases only which: Might become resectable after induction chemotherapy	Maximum tumour shrinkage	Three or four drug combination
2	Multiple metastases/sites, with: Rapid progression and/or Tumour-related symptoms/risk of rapid deterioration	Immediate clinically relevant response or at least tumour control	Three or four drug combination
3	Multiple metastases/sites without option for resection and no major symptoms or severe comorbidity	Abrogation of further progression Tumour shrinkage less relevant Low toxicity essential	Consider sequential approach: start with Single agent, or Doublet with low toxicity

ESMO: European Society for Medical Oncology.

some benefit[25,28].

There is no baseline predictive marker for the available anti-angiogenic drugs *e.g.*, bevacizumab or aflibercept. Changes in levels of angiogenic factors (*e.g.*, basic fibroblast, placental, or hepatocyte growth factor) during treatment with bevacizumab might indicate development of resistance and predict progression[29,30]. However, as recently shown in two randomized phase Ⅲ trials resistance to chemotherapy occurs before resistance to bevacizumab[31,32].

Beside the prediction of treatment toxicity (dihydropyrimidine-dehydrogenase deficiency for fluoropyrimidines or uridine-glucuronosyltransferase (UGT1A1) polymorphism for irinotecan), drug efficacy (*e.g.*, by topoisomerase-1 overexpression for irinotecan, or excision repair cross-complementing gene 1 polymorphisms for oxaliplatin) cannot be reliably predicted[33-37].

Current research focuses on distinct subsets of CRC patients defined by gene arrays, epigenetic alterations, or cancer stem cells, which might allow for a better treatment stratification[38-42]. Moreover, liquid biopsies (either by analysis of circulating DNA or tumour cells) obtained during course of treatment might give insights into tumour changes and development of resistance[43-46].

STRATIFICATION OF FIRST LINE MANAGEMENT FOR MCRC

Decision of treatment intensity for first line treatment should be based on clinical presentation at diagnosis, considering factors like patients' characteristics independent from the malignant disease, (if given) tumour-related symptoms, patients´ preferences, localisations of metastases, and the general treatment aim. Current ESMO guidelines stratify patients according to these factors in clinical groups with different treatment intensities (Table 2)[10]. Four groups are defined: ESMO group 0 comprising patients with clearly resectable liver metastases, group 1 with potentially resectable disease after achieving tumour response, group 2 symptomatic patients or high tumour load with risk of rapid deterioration and finally group 3 with asymptomatic, low tumour burden and severe co-morbidity.

For ESMO group 0 patients with clearly R0 resectable colorectal liver metastases surgery is the treatment of choice due to the proven chance of cure, whereas the sequence and intensity of perioperative chemotherapy is controversial. Based on the current ESMO consensus these patients should be managed preferably by perioperative FOLFOX for 3 mo before and 3 mo after resection[10,47,48]. Alternatively upfront resection with or without postoperative chemotherapy might be applied, particularly in metachronous, small and single liver metastasis[10]. Although intensification of perioperative treatment with antibodies has shown feasibility in single arm phase Ⅱ trials (*e.g.*, for bevacizumab), recently reported preliminary results of the New EPOC trial, evaluating chemotherapy and cetuximab in the perioperative setting, have raised strong scepticism[49,50]. Therefore, FOLFOX currently remains the standard treatment for clearly resectable liver metastases.

Patients with unresectable disease (ESMO groups 1, 2 or 3) should receive upfront systemic chemotherapy, apart from the small group of asymptomatic patients with low tumour burden eligible for and complying with a watch and wait approach[51,52]. Whereas groups 1 and 2 patients urge for intensive upfront chemotherapy to either ensure secondary resectability or allow for rapid symptom control, group 3 could be treated with a sequential treatment approach, starting with a low toxic single agent or two-drug combination regimen. Patients with asymptomatic, but surely unresectable disease due to location or overall extent and without relevant co-morbidity may not be ideally stratified in ESMO group 3, but rather treated with upfront intensive chemotherapy. Moreover, current available phase Ⅲ trials included patients irrespective of ESMO grouping, thus limiting the potential prognostic or predictive value of upfront patient stratification. Although grouping patients might be helpful for guidance of treatment strategy beyond induction treatment, *e.g.*, secondary resection, main systemic treatment options are either intensive three to four drug regimens or "sequential" one to two drugs regimens (Table 3).

SELECTION OF AN INTENSIVE FIRST LINE REGIMEN FOR MCRC

With respect to the increasing awareness of secondary surgery and developments in surgical and locally ablative measures, there is a growing group of patients that might

Table 3 Available treatment regimens for first-line metastatic colorectal cancer

Treatment intensity	Molecular factor	Regimens
Single agent		5FU/LV
		Capecitabin
Two-drug		Capecitabin/bevacizumab
		FOLFOX/XELOX
		FOLFIRI/XELIRI
Three-drug	*RAS* wt	FOLFOX + panitumumab
		FOLFIRI + cetuximab
	Independent of RAS status	FOLFOX/XELOX + bevacizumab
		FOLFIRI/XELIRI + bevacizumab
		FOLFOXIRI
Four-drug		FOLFOXIRI + bevacizumab

Combination chemotherapy with 5-fluorouracil, folinic acid (5FU/LV), and oxaliplatin (FOLFOX), or irinotecan (FOLFIRI) or both (FOLFOXIRI), or capecitabine and oxaliplatin (XELOX) or irinotecan (XELIRI).

be converted to resectability or at least achieve a "no evidence of disease" status after integration of other ablative techniques, and thus benefit from intensive upfront treatment. Therefore, either a chemotherapy doublet in combination with the VEGF antibody (bevacizumab) or an EGFR antibody [only *RAS* wild-type patients], or a chemo triplet (FOLFOXIRI) and more recently the highly active four drug regimen [FOLFOXIRI and bevacizumab or similar combinations (*e.g.*, FOLFIRINOX with a 5FU Bolus and slightly different doses) with EGFR antibodies] are available treatment options in this situation[4,22,53-59]. Comparative quantity, quality and celerity of response of these regimens are a matter of debate and currently only limited randomized data are available.

Preliminary data of the phase Ⅱ PEAK study comparing FOLFOX in combination with either panitumumab or bevacizumab in 285 previously untreated, *KRAS* wild-type mCRC patients indicated similar overall response rate (ORR)[60]. In the all *RAS* wildtype (*KRAS*/*NRAS* exon 2, 3 and 4) population panitumumab and FOLFOX significantly prolonged PFS (13.1 mo *vs* 9.5 mo, HR = 0.63; 95%CI: 0.43-0.94, $P = 0.02$) and showed a favourable trend in OS (HR = 0.55; $P = 0.06$) compared to bevacizumab and FOLFOX[61]. Similarly, early results from the phase Ⅲ AIO KRK-0306 (FIRE 3) study comparing FOLFIRI with either bevacizumab or cetuximab in 592 *KRAS* wildtype patients demonstrated a significantly prolonged OS (28.7 mo *vs* 25 mo, HR = 0.77; 95%CI: 0.62-0.96, $P = 0.017$) besides similar ORR (62% *vs* 58%, $P = 0.183$) and PFS (10 mo *vs* 10.3 mo, HR = 1.06; 95%CI: 0.88-1.26, $P = 0.547$) for cetuximab *vs* bevacizumab based chemotherapy, respectively[3]. Recent analyses demonstrated a pronounced OS benefit in *RAS* wildtype patients (33.1 mo *vs* 25.9 mo, $P = 0.01$) in favour of the cetuximab combination[62]. Subsequent treatments were balanced in regard of use of second line oxaliplatin and the cross over to the other antibody (46.6% receiving bevacizumab after cetuximab and 41.4% receiving EGFR antibody after bevacizumab). Interestingly, treatment in the cetuximab arm was shorter with a median duration of 4.8 mo *vs* 5.3 mo for all drugs and 6.8 mo *vs* 8 mo for any drug compared to the bevacizumab arm respectively. Although the primary endpoint of the FIRE 3 trial (ORR) was not reached and results of both trials are not fully published, the similar trend in the FIRE 3 and the PEAK study suggest a beneficial impact for EGFR antibodies and chemotherapy in first line *RAS* wildtype mCRC. Further data will soon be available from the large Intergroup trial (CALGB/SWOG 80405).

Feasibility and efficacy of a maximum intensive treatment with a four-drug regimen has been preliminarily shown in the phase Ⅲ TRIBE trial comparing FOLFIRI/bevacizumab and FOLFOXIRI/bevaciumab[7]. Overall response rate 53% *vs* 65% ($P = 0.006$), PFS 9.7 mo *vs* 12.1 mo (HR = 0.75; 95%CI: 0.62-0.90, $P = 0.003$) and OS 25.8 mo *vs* 31.0 mo (HR = 0.79; 95%CI: 0.63-1.00, $P = 0.054$) favoured the FOLFOXIRI and bevacizumab arm. Secondary surgery was applied at similar rates in both arms (12% *vs* 15% with the four-drug regimen). Treatment was generally well tolerated. Although rates of distinct grade 3/4 toxicity, particular, diarrhoea (11% *vs* 19%), stomatitis (4% *vs* 9%) and neutropenia (20% *vs* 50%) were significantly higher with the four-drug regimen, rates of febrile neutropenia, severe adverse events and treatment related death were similar. Efficacy of FOLFOXIRI and bevacizumab was independent of *KRAS* mutational status. Interestingly, patients with BRAF mutations seem to have better outcome with the four-drug regimen, despite their poor prognosis. In regard of similar outcomes in non-randomized phase Ⅱ trials FOLFOXIRI/bevacizumab should be considered for BRAF mutated patients[63,64].

According to the most recently presented preliminary trial results, the choice of first line regimen, *e.g.*, FOLFIRI + cetuximab (or FOLFOX + panitumumab) for *RAS* wildtype patients or FOLFOXIRI + bevacizumab for patients with good performance status seems to be relevant for the achievement of an OS of about 2.5 years[3,7]. Available treatment options are summarized in Table 4.

SELECTION OF A NON-INTENSE OR SEQUENTIAL TREATMENT APPROACH FOR MCRC

An increasingly ageing population with related co-morbidity which might not be amenable for a secondary curative approach (ESMO group 3) urge for comprehensive assessments focusing on toxicity and outcome prediction and well tolerated regimens for these patients (*e.g.*, single agent or two drug combinations)[15,65]. In the recently reported phase Ⅲ AVEX trial the addition of bevacizumab to capecitabine prolonged PFS from 5.1 to 9.1 mo (HR = 0.53; 95%CI: 0.41-0.69, $P < 0.0001$) and showed a strong trend in OS with an acceptable tolerability profile in patients with at least 70 years of age[66]. Alternatively, upfront combination with fluoropyrimidines and oxaliplatin seems to be feasible in elderly patients and prefer-

Table 4 Efficacy and tolerability of three to four drug first line regimen

Regimen	Efficacy				Tolerability		
	PFS		OS		Grade 3/4 AE	SAE	Fatal AEs
	RAS wt	RAS mut	RAS wt	RAS mut			
FOLFOX + panitumumab[25]	10.1	7.3[1]	25.8	15.5[1]	84%	40%	5%
FOLFIRI + cetuximab[4,62]	10.5	NR[1]	33.1	NR[1]	71%-79%	26%	NR
	9.9 (KRAS exon 2)		23.5 (KRAS exon 2)				
FOLFOX/XELOX + bevacizumab[56]	9.4		21.3		80%	NR	2%
FOLFIRI + bevacizumab[7,62]	10.4	NR	25.9	NR	NR	20%	3.5%
	9.7		25.8				
FOLFOXIRI + bevacizumab[7]	12.1		31.0		NR	20%	2.8%

[1]These fields only informative (epidermal growth factor receptor antibodies not licensed for *RAS* mutated tumours). Combination chemotherapy with 5-fluorouracil, folinic acid, and oxaliplatin (FOLFOX), or irinotecan (FOLFIRI) or both (FOLFOXIRI), or capecitabine and oxaliplatin (XELOX). PFS: Progression free survival; OS: Overall survival; AE: Adverse events; SAE: Severe adverse events; NR: Not reported.

ably, if applied with dose reductions, compared to singe agent fluoropyrimidine alone[15,67]. However, for elderly patients a tolerable and efficacious first line regimen seem to be particulary relevant, with less than 50% of patients receiving second line treatment.

Sequential treatment strategies were evaluated independent of age in first line mCRC[66,68-70]. Although sequential treatment did not seem to be inferior to upfront two-drug combination in trials of the chemotherapy only era (only fluoropyrimidines, irinotecan and oxaliplatin), it is questionable whether these results can be transferred into the current treatment situation (including molecular targeting agents)[68-70].

LIMITATIONS FOR CHEMOTHERAPY AND ANTIBODY COMBINATIONS

Besides very few limitations antibodies can be combined with fluoropyrimidines, oxaliplatin and/or irinotecan in several combinations. EGFR antibodies and bevacizumab should not be combined[71,72]. If EGFR antibodies are combined with an oxaliplatin based chemotherapy backbone, infusional 5FU (FOLFOX) should be chosen instead of an oral or bolus fluoropyrimidine regimen (XELOX or FLOX) according to clinical data from the COIN and NORDIC VII studies showing no benefit for the addition of cetuximab to these regimen[22,73].

The combination of capecitabin and irinotecan (with or without oxaliplatin or bevacizumab) requires dose reductions for both drugs[74-76]. Similarly, FOLFOXIRI needs to be dose reduced in combination with EGFR antibodies[58,59].

ADDITION OF UPFRONT LOCAL TREATMENT IN UNRESECTABLE MCRC PATIENTS

Integration of secondary resection after response to induction chemotherapy is a well-established treatment approach[8,77]. The randomized CLOCC trial furthermore showed that upfront local ablation by radiofrequency with or without liver surgery followed by chemotherapy in patients with unresectable liver metastases was beneficial in terms of PFS (16.8 mo *vs* 9.9 mo, $P = 0.025$) compared to chemotherapy alone[78]. Comparative data comparing upfront with post-induction local ablation are not available. However, post-induction ablation likely offers a more stratified approach adapting for the individual patient and tumour biology and might thus be preferred.

CONCLUSION

Treatment of mCRC is complex and highly individualized taking into account disease and patient characteristics, molecular and biochemical markers and thus enabling a personalized management in terms of selecting the most appropriate measures and sequences of systemic and local treatment.

In regard of the current data unresectable patients with *RAS* wildtype should receive an EGFR antibody based chemotherapy, whereas patients with *RAS* mutation should receive two or three drug chemotherapy in combination with bevacizumab, if an intensive treatment approach is chosen. For patients with a non-intense or sequential approach fluoropyrimidine and bevacizumab seems to be an efficacious and low toxic treatment option.

Future research might help to further tailor anti EGFR treatment, excluding patients deriving no benefit from EGFR inhibition. Moreover, close meshed and timely information (*e.g.*, acquired by liquid biopsies) about the current molecular tumour situation and potentially developing resistance might be helpful to guide treatment during the course of disease.

REFERENCES

1 **Jemal A**, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69-90 [PMID: 21296855 DOI: 10.3322/caac.20107]

2 **Malvezzi M**, Bertuccio P, Levi F, La Vecchia C, Negri E. European cancer mortality predictions for the year 2012. *Ann Oncol* 2012; **23**: 1044-1052 [PMID: 22373539 DOI: 10.1093/annonc/mds024]

3 **Stintzing S**, Fischer von Weikersthal L, Decker T, Vehling-Kaiser U, Jäger E, Heintges T, Stoll C, Giessen C, Modest DP, Neumann J, Jung A, Kirchner T, Scheithauer W, Heinemann V. FOLFIRI plus cetuximab versus FOLFIRI plus bevacizumab as first-line treatment for patients with metastatic colorectal cancer-subgroup analysis of patients with KRAS: mutated tumours in the randomised German AIO study KRK-0306. *Ann Oncol* 2012; **23**: 1693-1699 [PMID: 22219013]

4 **Van Cutsem E**, Köhne CH, Láng I, Folprecht G, Nowacki MP, Cascinu S, Shchepotin I, Maurel J, Cunningham D, Tejpar S, Schlichting M, Zubel A, Celik I, Rougier P, Ciardiello F. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. *J Clin Oncol* 2011; **29**: 2011-2019 [PMID: 21502544 DOI: 10.1200/JCO.2010.33.5091]

5 **Douillard JY**, Siena S, Cassidy J, Tabernero J, Burkes R, Barugel M, Humblet Y, Bodoky G, Cunningham D, Jassem J, Rivera F, Kocákova I, Ruff P, Błasińska-Morawiec M, Šmakal M, Canon JL, Rother M, Oliner KS, Wolf M, Gansert J. Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. *J Clin Oncol* 2010; **28**: 4697-4705 [PMID: 20921465 DOI: 10.1200/JCO.2009.27.4860]

6 **Kopetz S**, Chang GJ, Overman MJ, Eng C, Sargent DJ, Larson DW, Grothey A, Vauthey JN, Nagorney DM, McWilliams RR. Improved survival in metastatic colorectal cancer is associated with adoption of hepatic resection and improved chemotherapy. *J Clin Oncol* 2009; **27**: 3677-3683 [PMID: 19470929 DOI: 10.1200/JCO.2008.20.5278]

7 **Falcone A**, Cremolini C, Masi G. FOLFOXIRI/bevacizumab (bev) versus FOLFIRI/bev as first-line treatment in unresectable metastatic colorectal cancer (mCRC) patients (pts): Results of the phase III TRIBE trial by GONO group. *J Clin Oncol* 2013; **31**suppl: 3505 [abstract]

8 **Folprecht G**, Gruenberger T, Bechstein WO, Raab HR, Lordick F, Hartmann JT, Lang H, Frilling A, Stoehlmacher J, Weitz J, Konopke R, Stroszczynski C, Liersch T, Ockert D, Herrmann T, Goekkurt E, Parisi F, Köhne CH. Tumour response and secondary resectability of colorectal liver metastases following neoadjuvant chemotherapy with cetuximab: the CELIM randomised phase 2 trial. *Lancet Oncol* 2010; **11**: 38-47 [PMID: 19942479 DOI: 10.1016/S1470-2045(09)70330-4]

9 **Morris EJ**, Forman D, Thomas JD, Quirke P, Taylor EF, Fairley L, Cottier B, Poston G. Surgical management and outcomes of colorectal cancer liver metastases. *Br J Surg* 2010; **97**: 1110-1118 [PMID: 20632280 DOI: 10.1002/bjs.7032]

10 **Schmoll HJ**, Van Cutsem E, Stein A, Valentini V, Glimelius B, Haustermans K, Nordlinger B, van de Velde CJ, Balmana J, Regula J, Nagtegaal ID, Beets-Tan RG, Arnold D, Ciardiello F, Hoff P, Kerr D, Köhne CH, Labianca R, Price T, Scheithauer W, Sobrero A, Tabernero J, Aderka D, Barroso S, Bodoky G, Douillard JY, El Ghazaly H, Gallardo J, Garin A, Glynne-Jones R, Jordan K, Meshcheryakov A, Papamichail D, Pfeiffer P, Souglakos I, Turhal S, Cervantes A. ESMO Consensus Guidelines for management of patients with colon and rectal cancer. a personalized approach to clinical decision making. *Ann Oncol* 2012; **23**: 2479-2516 [PMID: 23012255 DOI: 10.1093/annonc/mds236]

11 **Ren J**, Li G, Ge J, Li X, Zhao Y. Is K-ras gene mutation a prognostic factor for colorectal cancer: a systematic review and meta-analysis. *Dis Colon Rectum* 2012; **55**: 913-923 [PMID: 22810479]

12 **Price TJ**, Hardingham JE, Lee CK, Weickhardt A, Townsend AR, Wrin JW, Chua A, Shivasami A, Cummins MM, Murone C, Tebbutt NC. Impact of KRAS and BRAF Gene Mutation Status on Outcomes From the Phase III AGITG MAX Trial of Capecitabine Alone or in Combination With Bevacizumab and Mitomycin in Advanced Colorectal Cancer. *J Clin Oncol* 2011; **29**: 2675-2682 [PMID: 21646616 DOI: 10.1200/JCO.2010.34.5520]

13 **Köhne CH**, Cunningham D, Di Costanzo F, Glimelius B, Blijham G, Aranda E, Scheithauer W, Rougier P, Palmer M, Wils J, Baron B, Pignatti F, Schöffski P, Micheel S, Hecker H. Clinical determinants of survival in patients with 5-fluorouracil-based treatment for metastatic colorectal cancer: results of a multivariate analysis of 3825 patients. *Ann Oncol* 2002; **13**: 308-317 [PMID: 11886010]

14 **Sorbye H**, Köhne CH, Sargent DJ, Glimelius B. Patient characteristics and stratification in medical treatment studies for metastatic colorectal cancer: a proposal for standardization of patient characteristic reporting and stratification. *Ann Oncol* 2007; **18**: 1666-1672 [PMID: 17660495 DOI: 10.1093/annonc/mdm267]

15 **Seymour MT**, Thompson LC, Wasan HS, Middleton G, Brewster AE, Shepherd SF, O'Mahony MS, Maughan TS, Parmar M, Langley RE. Chemotherapy options in elderly and frail patients with metastatic colorectal cancer (MRC FOCUS2): an open-label, randomised factorial trial. *Lancet* 2011; **377**: 1749-1759 [PMID: 21570111 DOI: 10.1016/S0140-6736(11)60399-1]

16 **Asghar U**, Hawkes E, Cunningham D. Predictive and prognostic biomarkers for targeted therapy in metastatic colorectal cancer. *Clin Colorectal Cancer* 2010; **9**: 274-281 [PMID: 21208841 DOI: 10.3816/CCC.2010.n.040]

17 **Cohen SJ**, Punt CJ, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY, Picus J, Morse M, Mitchell E, Miller MC, Doyle GV, Tissing H, Terstappen LW, Meropol NJ. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. *J Clin Oncol* 2008; **26**: 3213-3221 [PMID: 18591556 DOI: 10.1200/JCO.2007.15.8923]

18 **Karapetis CS**, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, Simes RJ, Chalchal H, Shapiro JD, Robitaille S, Price TJ, Shepherd L, Au HJ, Langer C, Moore MJ, Zalcberg JR. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med* 2008; **359**: 1757-1765 [PMID: 18946061 DOI: 10.1056/NEJMoa0804385]

19 **Amado RG**, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, Patterson SD, Chang DD. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol* 2008; **26**: 1626-1634 [PMID: 18316791 DOI: 10.1200/JCO.2007.14.7116]

20 **De Roock W**, Jonker DJ, Di Nicolantonio F, Sartore-Bianchi A, Tu D, Siena S, Lamba S, Arena S, Frattini M, Piessevaux H, Van Cutsem E, O'Callaghan CJ, Khambata-Ford S, Zalcberg JR, Simes J, Karapetis CS, Bardelli A, Tejpar S. Association of KRAS p.G13D mutation with outcome in patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab. *JAMA* 2010; **304**: 1812-1820 [PMID: 20978259 DOI: 10.1001/jama.2010.1535]

21 **Tejpar S**, Celik I, Schlichting M, Sartorius U, Bokemeyer C, Van Cutsem E. Association of KRAS G13D tumor mutations with outcome in patients with metastatic colorectal cancer treated with first-line chemotherapy with or without cetuximab. *J Clin Oncol* 2012; **30**: 3570-3577 [PMID: 22734028 DOI: 10.1200/JCO.2012.42.2592]

22 **Maughan TS**, Adams RA, Smith CG, Meade AM, Seymour MT, Wilson RH, Idziaszczyk S, Harris R, Fisher D, Kenny SL, Kay E, Mitchell JK, Madi A, Jasani B, James MD, Bridgewater J, Kennedy MJ, Claes B, Lambrechts D, Kaplan R, Cheadle JP. Addition of cetuximab to oxaliplatin-based first-line combination chemotherapy for treatment of advanced colorectal cancer: results of the randomised phase 3 MRC COIN trial. *Lancet* 2011; **377**: 2103-2114 [PMID: 21641636 DOI: 10.1016/S0140-6736(11)60613-2]

23 **Peeters M**, Douillard JY, Van Cutsem E, Siena S, Zhang K,

Williams R, Wiezorek J. Mutant KRAS codon 12 and 13 alleles in patients with metastatic colorectal cancer: assessment as prognostic and predictive biomarkers of response to panitumumab. *J Clin Oncol* 2013; **31**: 759-765 [PMID: 23182985 DOI: 10.1200/JCO.2012.45.1492]

24 **Seymour MT**, Brown SR, Middleton G, Maughan T, Richman S, Gwyther S, Lowe C, Seligmann JF, Wadsley J, Maisey N, Chau I, Hill M, Dawson L, Falk S, O'Callaghan A, Benstead K, Chambers P, Oliver A, Marshall H, Napp V, Quirke P. Panitumumab and irinotecan versus irinotecan alone for patients with KRAS wild-type, fluorouracil-resistant advanced colorectal cancer (PICCOLO): a prospectively stratified randomised trial. *Lancet Oncol* 2013; **14**: 749-759 [PMID: 23725851 DOI: 10.1016/S1470-2045(13)70163-3]

25 **Douillard JY**, Oliner KS, Siena S, Tabernero J, Burkes R, Barugel M, Humblet Y, Bodoky G, Cunningham D, Jassem J, Rivera F, Kocákova I, Ruff P, Błasińska-Morawiec M, Šmakal M, Canon JL, Rother M, Williams R, Rong A, Wiezorek J, Sidhu R, Patterson SD. Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. *N Engl J Med* 2013; **369**: 1023-1034 [PMID: 24024839 DOI: 10.1056/NEJMoa1305275]

26 **De Roock W**, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, Kalogeras KT, Kotoula V, Papamichael D, Laurent-Puig P, Penault-Llorca F, Rougier P, Vincenzi B, Santini D, Tonini G, Cappuzzo F, Frattini M, Molinari F, Saletti P, De Dosso S, Martini M, Bardelli A, Siena S, Sartore-Bianchi A, Tabernero J, Macarulla T, Di Fiore F, Gangloff AO, Ciardiello F, Pfeiffer P, Qvortrup C, Hansen TP, Van Cutsem E, Piessevaux H, Lambrechts D, Delorenzi M, Tejpar S. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. *Lancet Oncol* 2010; **11**: 753-762 [PMID: 20619739 DOI: 10.1016/S1470-2045(10)70130-3]

27 **Di Nicolantonio F**, Martini M, Molinari F, Sartore-Bianchi A, Arena S, Saletti P, De Dosso S, Mazzucchelli L, Frattini M, Siena S, Bardelli A. Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *J Clin Oncol* 2008; **26**: 5705-5712 [PMID: 19001320 DOI: 10.1200/JCO.2008.18.0786]

28 **Bokemeyer C**, Van Cutsem E, Rougier P, Ciardiello F, Heeger S, Schlichting M, Celik I, Köhne CH. Addition of cetuximab to chemotherapy as first-line treatment for KRAS wild-type metastatic colorectal cancer: pooled analysis of the CRYSTAL and OPUS randomised clinical trials. *Eur J Cancer* 2012; **48**: 1466-1475 [PMID: 22446022 DOI: 10.1016/j.ejca.2012.02.057]

29 **Kopetz S**, Hoff PM, Morris JS, Wolff RA, Eng C, Glover KY, Adinin R, Overman MJ, Valero V, Wen S, Lieu C, Yan S, Tran HT, Ellis LM, Abbruzzese JL, Heymach JV. Phase II trial of infusional fluorouracil, irinotecan, and bevacizumab for metastatic colorectal cancer: efficacy and circulating angiogenic biomarkers associated with therapeutic resistance. *J Clin Oncol* 2010; **28**: 453-459 [PMID: 20008624 DOI: 10.1200/JCO.2009.24.8252]

30 **Loupakis F**, Cremolini C, Fioravanti A, Orlandi P, Salvatore L, Masi G, Di Desidero T, Canu B, Schirripa M, Frumento P, Di Paolo A, Danesi R, Falcone A, Bocci G. Pharmacodynamic and pharmacogenetic angiogenesis-related markers of first-line FOLFOXIRI plus bevacizumab schedule in metastatic colorectal cancer. *Br J Cancer* 2011; **104**: 1262-1269 [PMID: 21407216 DOI: 10.1038/bjc.2011.85]

31 **Bennouna J**, Sastre J, Arnold D, Österlund P, Greil R, Van Cutsem E, von Moos R, Viéitez JM, Bouché O, Borg C, Steffens CC, Alonso-Orduña V, Schlichting C, Reyes-Rivera I, Bendahmane B, André T, Kubicka S. Continuation of bevacizumab after first progression in metastatic colorectal cancer (ML18147): a randomised phase 3 trial. *Lancet Oncol* 2013; **14**: 29-37 [PMID: 23168366 DOI: 10.1016/S1470-2045(12)70477-1]

32 **Masi G**, Loupakis F, Salvatore L, Cremolini C. A randomized phase III study evaluating the continuation of bevacizumab (BV) beyond progression in metastatic colorectal cancer (mCRC) patients (pts) who received BV as part of first-line treatment: results of the BEBYP trial by the Gruppo Oncologico Nord Ovest (GONO) [abstract]. *ESMO* 2012; **2012**: LBA 17

33 **Braun MS**, Richman SD, Quirke P, Daly C, Adlard JW, Elliott F, Barrett JH, Selby P, Meade AM, Stephens RJ, Parmar MK, Seymour MT. Predictive biomarkers of chemotherapy efficacy in colorectal cancer: results from the UK MRC FOCUS trial. *J Clin Oncol* 2008; **26**: 2690-2698 [PMID: 18509181 DOI: 10.1200/JCO.2007.15.5580]

34 **Koopman M**, Knijn N. The correlation between Topoisomerase-I (Topo1) expression and outcome of treatment with capecitabine and irinotecan in advanced colorectal cancer (ACC) patients (pts) treated in the CAIRO study of the Dutch Colorectal Cancer Group (DCCG). *Eur J Canc Care* 2009; **7**: 321

35 **Koopman M**, Venderbosch S, van Tinteren H, Ligtenberg MJ, Nagtegaal I, Van Krieken JH, Punt CJ. Predictive and prognostic markers for the outcome of chemotherapy in advanced colorectal cancer, a retrospective analysis of the phase III randomised CAIRO study. *Eur J Cancer* 2009; **45**: 1999-2006 [PMID: 19457654 DOI: 10.1016/j.ejca.2009.04.017]

36 **Boige V**, Mendiboure J, Pignon JP, Loriot MA, Castaing M, Barrois M, Malka D, Trégouët DA, Bouché O, Le Corre D, Miran I, Mulot C, Ducreux M, Beaune P, Laurent-Puig P. Pharmacogenetic assessment of toxicity and outcome in patients with metastatic colorectal cancer treated with LV5FU2, FOLFOX, and FOLFIRI: FFCD 2000-05. *J Clin Oncol* 2010; **28**: 2556-2564 [PMID: 20385995 DOI: 10.1200/JCO.2009.25.2106]

37 **Bokemeyer C**, Koehne C-H, Bondarenko I. Treatment outcome according to tumor ERCC1 expression status in OPUS study patients with metastatic colorectal cancer (mCRC) randomized to FOLFOX4 with/without cetuximab [abstract]. *J Clin Oncol* 2013; **31** suppl: 3537

38 **Crea F**, Fornaro L, Paolicchi E, Masi G, Frumento P, Loupakis F, Salvatore L, Cremolini C, Schirripa M, Graziano F, Ronzoni M, Ricci V, Farrar WL, Falcone A, Danesi R. An EZH2 polymorphism is associated with clinical outcome in metastatic colorectal cancer patients. *Ann Oncol* 2012; **23**: 1207-1213 [PMID: 21926398 DOI: 10.1093/annonc/mdr387]

39 **Sadanandam A**, Lyssiotis CA, Homicsko K, Collisson EA, Gibb WJ, Wullschleger S, Ostos LC, Lannon WA, Grotzinger C, Del Rio M, Lhermitte B, Olshen AB, Wiedenmann B, Cantley LC, Gray JW, Hanahan D. A colorectal cancer classification system that associates cellular phenotype and responses to therapy. *Nat Med* 2013; **19**: 619-625 [PMID: 23584089 DOI: 10.1038/nm.3175]

40 **Popovici V**, Budinska E, Tejpar S, Weinrich S, Estrella H, Hodgson G, Van Cutsem E, Xie T, Bosman FT, Roth AD, Delorenzi M. Identification of a poor-prognosis BRAF-mutant-like population of patients with colon cancer. *J Clin Oncol* 2012; **30**: 1288-1295 [PMID: 22393095 DOI: 10.1200/JCO.2011.39.5814]

41 **Ebert MP**, Tänzer M, Balluff B, Burgermeister E, Kretzschmar AK, Hughes DJ, Tetzner R, Lofton-Day C, Rosenberg R, Reinacher-Schick AC, Schulmann K, Tannapfel A, Hofheinz R, Röcken C, Keller G, Langer R, Specht K, Porschen R, Stöhlmacher-Williams J, Schuster T, Ströbel P, Schmid RM. TFAP2E-DKK4 and chemoresistance in colorectal cancer. *N Engl J Med* 2012; **366**: 44-53 [PMID: 22216841 DOI: 10.1056/NEJMoa1009473]

42 **Pohl A**, El-Khoueiry A, Yang D, Zhang W, Lurje G, Ning Y, Winder T, Hu-Lieskoven S, Iqbal S, Danenberg KD, Kahn M, Teo JL, Shriki J, Stebbing J, Lenz HJ. Pharmacogenetic profiling of CD133 is associated with response rate (RR) and progression-free survival (PFS) in patients with metastatic colorectal cancer (mCRC), treated with bevacizumab-based chemotherapy. *Pharmacogenomics J* 2013; **13**: 173-180 [PMID:

22231565 DOI: 10.1038/tpj.2011.61]
43 **Diaz LA**, Williams RT, Wu J, Kinde I, Hecht JR, Berlin J, Allen B, Bozic I, Reiter JG, Nowak MA, Kinzler KW, Oliner KS, Vogelstein B. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. *Nature* 2012; **486**: 537-540 [PMID: 22722843 DOI: 10.1038/nature11219]
44 **Heitzer E**, Auer M, Gasch C, Pichler M, Ulz P, Hoffmann EM, Lax S, Waldispuehl-Geigl J, Mauermann O, Lackner C, Höfler G, Eisner F, Sill H, Samonigg H, Pantel K, Riethdorf S, Bauernhofer T, Geigl JB, Speicher MR. Complex tumor genomes inferred from single circulating tumor cells by array-CGH and next-generation sequencing. *Cancer Res* 2013; **73**: 2965-2975 [PMID: 23471846]
45 **Crowley E**, Di Nicolantonio F, Loupakis F, Bardelli A. Liquid biopsy: monitoring cancer-genetics in the blood. *Nat Rev Clin Oncol* 2013; **10**: 472-484 [PMID: 23836314 DOI: 10.1038/nrclinonc.2013.110]
46 **Misale S**, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, Valtorta E, Schiavo R, Buscarino M, Siravegna G, Bencardino K, Cercek A, Chen CT, Veronese S, Zanon C, Sartore-Bianchi A, Gambacorta M, Gallicchio M, Vakiani E, Boscaro V, Medico E, Weiser M, Siena S, Di Nicolantonio F, Solit D, Bardelli A. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. *Nature* 2012; **486**: 532-536 [PMID: 22722830 DOI: 10.1038/nature11156]
47 **Nordlinger B**, Sorbye H, Glimelius B, Poston GJ, Schlag PM, Rougier P, Bechstein WO, Primrose JN, Walpole ET, Finch-Jones M, Jaeck D, Mirza D, Parks RW, Collette L, Praet M, Bethe U, Van Cutsem E, Scheithauer W, Gruenberger T. Perioperative chemotherapy with FOLFOX4 and surgery versus surgery alone for resectable liver metastases from colorectal cancer (EORTC Intergroup trial 40983): a randomised controlled trial. *Lancet* 2008; **371**: 1007-1016 [PMID: 18358928]
48 **Nordlinger B**, Sorbye H. EORTC liver metastases intergroup randomized phase III study 40983: Long-term survival results [abstract]. *J Clin Oncol* 2012; **30** suppl: 3508
49 **Primrose J**, Falk S, Finch-Jones M, Valle J, Sherlock D. A randomized clinical trial of chemotherapy compared to chemotherapy in combination with cetuximab in k-RAS wild-type patients with operable metastases from colorectal cancer: The new EPOC study [abstract]. *J Clin Oncol* 2013; **31** suppl: 3504
50 **Gruenberger B**, Tamandl D, Schueller J, Scheithauer W, Zielinski C, Herbst F, Gruenberger T. Bevacizumab, capecitabine, and oxaliplatin as neoadjuvant therapy for patients with potentially curable metastatic colorectal cancer. *J Clin Oncol* 2008; **26**: 1830-1835 [PMID: 18398148]
51 **Voskoboynik M**, Bae S, Ananda S, Desai J, Kosmider S, Gibbs P. An initial watch and wait approach is a valid strategy for selected patients with newly diagnosed metastatic colorectal cancer. *Ann Oncol* 2012; **23**: 2633-2637 [PMID: 22734008 DOI: 10.1093/annonc/mds184]
52 **Ackland SP**, Jones M, Tu D, Simes J, Yuen J, Sargeant AM, Dhillon H, Goldberg RM, Abdi E, Shepherd L, Moore MJ. A meta-analysis of two randomised trials of early chemotherapy in asymptomatic metastatic colorectal cancer. *Br J Cancer* 2005; **93**: 1236-1243 [PMID: 16265352 DOI: 10.1038/sj.bjc.6602841]
53 **Douillard JY**, Siena, S. Final results from PRIME: Randomized phase III study of panitumumab (pmab) with FOLFOX4 for first line metastatic colorectal cancer (mCRC). *J Clin Oncol* 2011; **29** suppl: 3510 [abstract]
54 **Falcone A**, Ricci S, Brunetti I, Pfanner E, Allegrini G, Barbara C, Crinò L, Benedetti G, Evangelista W, Fanchini L, Cortesi E, Picone V, Vitello S, Chiara S, Granetto C, Porcile G, Fioretto L, Orlandini C, Andreuccetti M, Masi G. Phase III trial of infusional fluorouracil, leucovorin, oxaliplatin, and irinotecan (FOLFOXIRI) compared with infusional fluorouracil, leucovorin, and irinotecan (FOLFIRI) as first-line treatment for metastatic colorectal cancer: the Gruppo Oncologico Nord Ovest. *J Clin Oncol* 2007; **25**: 1670-1676 [PMID: 17470860]
55 **Loupakis F**, Cremolini C, Masi G. FOLFOXIRI plus bevacizumab (bev) versus FOLFIRI plus bev as first-line treatment of metastatic colorectal cancer (MCRC): Results of the phase III randomized TRIBE trial [abstract]. *J Clin Oncol* 2013; **30** suppl 34: 336
56 **Saltz LB**, Clarke S, Díaz-Rubio E, Scheithauer W, Figer A, Wong R, Koski S, Lichinitser M, Yang TS, Rivera F, Couture F, Sirzén F, Cassidy J. Bevacizumab in combination with oxaliplatin-based chemotherapy as first-line therapy in metastatic colorectal cancer: a randomized phase III study. *J Clin Oncol* 2008; **26**: 2013-2019 [PMID: 18421054]
57 **Assenat E**, Desseigne F, Thezenas S, Viret F, Mineur L, Kramar A, Samalin E, Portales F, Bibeau F, Crapez-Lopez E, Bleuse JP, Ychou M. Cetuximab plus FOLFIRINOX (ERBIRINOX) as first-line treatment for unresectable metastatic colorectal cancer: a phase II trial. *Oncologist* 2011; **16**: 1557-1564 [PMID: 22016477 DOI: 10.1634/theoncologist.2011-0141]
58 **Garufi C**, Torsello A, Tumolo S, Ettorre GM, Zeuli M, Campanella C, Vennarecci G, Mottolese M, Sperduti I, Cognetti F. Cetuximab plus chronomodulated irinotecan, 5-fluorouracil, leucovorin and oxaliplatin as neoadjuvant chemotherapy in colorectal liver metastases: POCHER trial. *Br J Cancer* 2010; **103**: 1542-1547 [PMID: 20959822 DOI: 10.1038/sj.bjc.6605940]
59 **Fornaro L**, Lonardi S, Masi G, Loupakis F, Bergamo F, Salvatore L, Cremolini C, Schirripa M, Vivaldi C, Aprile G, Zaniboni A, Bracarda S, Fontanini G, Sensi E, Lupi C, Morvillo M, Zagonel V, Falcone A. FOLFOXIRI in combination with panitumumab as first-line treatment in quadruple wild-type (KRAS, NRAS, HRAS, BRAF) metastatic colorectal cancer patients: a phase II trial by the Gruppo Oncologico Nord Ovest (GONO). *Ann Oncol* 2013; **24**: 2062-2067 [PMID: 23666916 DOI: 10.1093/annonc/mdt165]
60 **Schwartzberg L**, Rivera-Herreros F, Karthaus M. A Randomized Phase 2 Study of mFOLFOX6 With Either Panitumumab or Bevacizumab as 1st-line Treatment in Patients With Unresectable Wild-type KRAS Metastatic Colorectal Cancer (mCRC) [abstract]. *J Clin Oncol* 2013; **30** suppl: 446
61 Schwartzberg L, Rivera-Herreros F, Karthaus M. Analysis of KRAS/NRAS mutations in PEAK: A randomized phase II study of FOLFOX6 plus panitumumab (pmab) or bevacizumab (bev) as first-line treatment (tx) for wild-type (WT) KRAS (exon 2) metastatic colorectal cancer (mCRC). *J Clin Oncol* 2013; **31** suppl: 3631 [abstract]
62 **Stintzing S**, Jung A, Rossius L. Analysis of KRAS/NRAS and BRAF mutations in FIRE-3: A randomized phase III study of FOLFIRI plus cetuximab or bevacizumab as first-line treatment for wild-type (WT) KRAS (exon 2) metastatic colorectal cancer (mCRC) patients. Available from: URL: http//eccamsterdam2013.ecco-org.eu/Scientific-Programme/Searchable-Programme.aspx#anchorScpr
63 **Masi G**, Loupakis F, Salvatore L, Fornaro L, Cremolini C, Cupini S, Ciarlo A, Del Monte F, Cortesi E, Amoroso D, Granetto C, Fontanini G, Sensi E, Lupi C, Andreuccetti M, Falcone A. Bevacizumab with FOLFOXIRI (irinotecan, oxaliplatin, fluorouracil, and folinate) as first-line treatment for metastatic colorectal cancer: a phase 2 trial. *Lancet Oncol* 2010; **11**: 845-852 [PMID: 20702138 DOI: 10.1016/S1470-2045(10)70175-3]
64 **Loupakis F**, Cremolini C, Salvatore L, Masi G, Sensi E, Schirripa M, Michelucci A, Pfanner E, Brunetti I, Lupi C, Antoniotti C, Bergamo F, Lonardi S, Zagonel V, Simi P, Fontanini G, Falcone A. FOLFOXIRI plus bevacizumab as first-line treatment in BRAF mutant metastatic colorectal cancer. *Eur J Cancer* 2014; **50**: 57-63
65 **Aparicio T**, Jouve JL, Teillet L, Gargot D, Subtil F, Le Brun-Ly V, Cretin J, Locher C, Bouché O, Breysacher G, Charneau J, Seitz JF, Gasmi M, Stefani L, Ramdani M, Lecomte T,

Mitry E. Geriatric factors predict chemotherapy feasibility: ancillary results of FFCD 2001-02 phase III study in first-line chemotherapy for metastatic colorectal cancer in elderly patients. *J Clin Oncol* 2013; **31**: 1464-1470 [PMID: 23460711 DOI: 10.1200/JCO.2012.42.9894]

66 **Cunningham D**, Lang I, Marcuello E, Lorusso V, Ocvirk J, Shin DB, Jonker D, Osborne S, Andre N, Waterkamp D, Saunders MP. Bevacizumab plus capecitabine versus capecitabine alone in elderly patients with previously untreated metastatic colorectal cancer (AVEX): an open-label, randomised phase 3 trial. *Lancet Oncol* 2013; **14**: 1077-1085 [PMID: 24028813 DOI: 10.1016/S1470-2045(13)70154-2]

67 **Goldberg RM**, Tabah-Fisch I, Bleiberg H, de Gramont A, Tournigand C, Andre T, Rothenberg ML, Green E, Sargent DJ. Pooled analysis of safety and efficacy of oxaliplatin plus fluorouracil/leucovorin administered bimonthly in elderly patients with colorectal cancer. *J Clin Oncol* 2006; **24**: 4085-4091 [PMID: 16943526 DOI: 10.1200/JCO.2006.06.9039]

68 **Koopman M**, Antonini NF, Douma J, Wals J, Honkoop AH, Erdkamp FL, de Jong RS, Rodenburg CJ, Vreugdenhil G, Loosveld OJ, van Bochove A, Sinnige HA, Creemers GJ, Tesselaar ME, Slee PH, Werter MJ, Mol L, Dalesio O, Punt CJ. Sequential versus combination chemotherapy with capecitabine, irinotecan, and oxaliplatin in advanced colorectal cancer (CAIRO): a phase III randomised controlled trial. *Lancet* 2007; **370**: 135-142 [PMID: 17630036 DOI: 10.1016/S0140-6736(07)61086-1]

69 **Seymour MT**, Maughan TS, Ledermann JA, Topham C, James R, Gwyther SJ, Smith DB, Shepherd S, Maraveyas A, Ferry DR, Meade AM, Thompson L, Griffiths GO, Parmar MK, Stephens RJ. Different strategies of sequential and combination chemotherapy for patients with poor prognosis advanced colorectal cancer (MRC FOCUS): a randomised controlled trial. *Lancet* 2007; **370**: 143-152 [PMID: 17630037 DOI: 10.1016/S0140-6736(07)61087-3]

70 **Cunningham D**, Sirohi B, Pluzanska A, Utracka-Hutka B, Zaluski J, Glynne-Jones R, Koralewski P, Bridgewater J, Mainwaring P, Wasan H, Wang JY, Szczylik C, Clingan P, Chan RT, Tabah-Fisch I, Cassidy J. Two different first-line 5-fluorouracil regimens with or without oxaliplatin in patients with metastatic colorectal cancer. *Ann Oncol* 2009; **20**: 244-250 [PMID: 18854549 DOI: 10.1093/annonc/mdn638]

71 **Hecht JR**, Mitchell E, Chidiac T, Scroggin C, Hagenstad C, Spigel D, Marshall J, Cohn A, McCollum D, Stella P, Deeter R, Shahin S, Amado RG. A randomized phase IIIB trial of chemotherapy, bevacizumab, and panitumumab compared with chemotherapy and bevacizumab alone for metastatic colorectal cancer. *J Clin Oncol* 2009; **27**: 672-680 [PMID: 19114685]

72 **Tol J**, Koopman M, Cats A, Rodenburg CJ, Creemers GJ, Schrama JG, Erdkamp FL, Vos AH, van Groeningen CJ, Sinnige HA, Richel DJ, Voest EE, Dijkstra JR, Vink-Börger ME, Antonini NF, Mol L, van Krieken JH, Dalesio O, Punt CJ. Chemotherapy, bevacizumab, and cetuximab in metastatic colorectal cancer. *N Engl J Med* 2009; **360**: 563-572 [PMID: 19196673]

73 **Tveit KM**, Guren T, Glimelius B, Pfeiffer P, Sorbye H, Pyrhonen S, Sigurdsson F, Kure E, Ikdahl T, Skovlund E, Fokstuen T, Hansen F, Hofsli E, Birkemeyer E, Johnsson A, Starkhammar H, Yilmaz MK, Keldsen N, Erdal AB, Dajani O, Dahl O, Christoffersen T. Phase III trial of cetuximab with continuous or intermittent fluorouracil, leucovorin, and oxaliplatin (Nordic FLOX) versus FLOX alone in first-line treatment of metastatic colorectal cancer: the NORDIC-VII study. *J Clin Oncol* 2012; **30**: 1755-1762 [PMID: 22473155 DOI: 10.1200/JCO.2011.38.0915]

74 **Köhne CH**, De Greve J, Hartmann JT, Lang I, Vergauwe P, Becker K, Braumann D, Joosens E, Müller L, Janssens J, Bokemeyer C, Reimer P, Link H, Späth-Schwalbe E, Wilke HJ, Bleiberg H, Van Den Brande J, Debois M, Bethe U, Van Cutsem E. Irinotecan combined with infusional 5-fluorouracil/folinic acid or capecitabine plus celecoxib or placebo in the first-line treatment of patients with metastatic colorectal cancer. EORTC study 40015. *Ann Oncol* 2008; **19**: 920-926 [PMID: 18065406]

75 **Zarate R**, Rodríguez J, Bandres E, Patiño-Garcia A, Ponz-Sarvise M, Viudez A, Ramirez N, Bitarte N, Chopitea A, Gacía-Foncillas J. Oxaliplatin, irinotecan and capecitabine as first-line therapy in metastatic colorectal cancer (mCRC): a dose-finding study and pharmacogenomic analysis. *Br J Cancer* 2010; **102**: 987-994 [PMID: 20216541 DOI: 10.1038/sj.bjc.6605595]

76 **Ducreux M**, Adenis A, Pignon JP, François E, Chauffert B, Ichanté JL, Boucher E, Ychou M, Pierga JY, Montoto-Grillot C, Conroy T. Efficacy and safety of bevacizumab-based combination regimens in patients with previously untreated metastatic colorectal cancer: final results from a randomised phase II study of bevacizumab plus 5-fluorouracil, leucovorin plus irinotecan versus bevacizumab plus capecitabine plus irinotecan (FNCLCC ACCORD 13/0503 study). *Eur J Cancer* 2013; **49**: 1236-1245 [PMID: 23352604 DOI: 10.1016/j.ejca.2012.12.011]

77 **Ye LC**, Liu TS, Ren L, Wei Y, Zhu DX, Zai SY, Ye QH, Yu Y, Xu B, Qin XY, Xu J. Randomized controlled trial of cetuximab plus chemotherapy for patients with KRAS wild-type unresectable colorectal liver-limited metastases. *J Clin Oncol* 2013; **31**: 1931-1938 [PMID: 23569301 DOI: 10.1200/JCO.2012.44.8308]

78 **Ruers T**, Punt C, Van Coevorden F, Pierie JP, Borel-Rinkes I, Ledermann JA, Poston G, Bechstein W, Lentz MA, Mauer M, Van Cutsem E, Lutz MP, Nordlinger B. Radiofrequency ablation combined with systemic treatment versus systemic treatment alone in patients with non-resectable colorectal liver metastases: a randomized EORTC Intergroup phase II study (EORTC 40004). *Ann Oncol* 2012; **23**: 2619-2626 [PMID: 22431703 DOI: 10.1093/annonc/mds053]

P- Reviewers: Crea F, Ji JF, Triantafyllou K **S- Editor**: Gou SX
L- Editor: A **E- Editor**: Wu HL

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.908

World J Gastroenterol 2014 January 28; 20(4): 908-922
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Inflammation and colorectal cancer, when microbiota-host mutualism breaks

Marco Candela, Silvia Turroni, Elena Biagi, Franck Carbonero, Simone Rampelli, Carla Fiorentini, Patrizia Brigidi

Marco Candela, Silvia Turroni, Elena Biagi, Simone Rampelli, Patrizia Brigidi, Department of Pharmacy and Biotechnology, University of Bologna, 40126 Bologna, Italy
Franck Carbonero, Department of Food Science, University of Arkansas, Fayetteville, AR 72704, United States
Carla Fiorentini, Department of Therapeutic Research and Medicines Evaluation, Istituto Superiore di Sanità, 00161 Rome, Italy
Author contributions: Candela M conceived and designed the article, and wrote the manuscript; Turroni S, Biagi E, Carbonero F, Fiorentini C reviewed literature, wrote the manuscript, revised and edited the draft; Rampelli S performed bioinformatics analysis; Brigidi P reviewed and edited the final version of the article; all Authors read and approved the final version of the manuscript.
Correspondence to: Marco Candela, PhD, Department of Pharmacy and Biotechnology, University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy. marco.candela@unibo.it
Telephone: +39-51-2099727 Fax: +39-51-2099734
Received: September 27, 2013 Revised: November 26, 2013
Accepted: December 12, 2013
Published online: January 28, 2014

Abstract

Structural changes in the gut microbial community have been shown to accompany the progressive development of colorectal cancer. In this review we discuss recent hypotheses on the mechanisms involved in the bacteria-mediated carcinogenesis, as well as the triggering factors favoring the shift of the gut microbiota from a mutualistic to a pro-carcinogenic configuration. The possible role of inflammation, bacterial toxins and toxic microbiota metabolites in colorectal cancer onset is specifically discussed. On the other hand, the strategic role of inflammation as the keystone factor in driving microbiota to become carcinogenic is suggested. As a common outcome of different environmental and endogenous triggers, such as diet, aging, pathogen infection or genetic predisposition, inflammation can compromise the microbiota-host mutualism, forcing the increase of pathobionts at the expense of health-promoting groups, and allowing the microbiota to acquire an overall pro-inflammatory configuration. Consolidating inflammation in the gut, and favoring the bloom of toxigenic bacterial drivers, these changes in the gut microbial ecosystem have been suggested as pivotal in promoting carcinogenesis. In this context, it will become of primary importance to implement dietary or probiotics-based interventions aimed at preserving the microbiota-host mutualism along aging, counteracting deviations that favor a pro-carcinogenic microbiota asset.

Key words: Colorectal cancer; Inflammation; Gut microbiome; Co-abundance groups

Core tip: By performing the co-abundance groups analysis of the publicly available datasets from microbiome surveys in colorectal cancer (CRC) patients, we have been successful in identifying pro-carcinogenic and protective groups of microorganisms, showing the potential to modulate the fate of CRC onset and progression. Possible mechanisms involved in microbiota-dependent carcinogenesis are reviewed, and the central role of inflammation as a trigger forcing the microbiota from a mutualistic configuration to a CRC-promoting asset is discussed. Finally, possible intervention strategies for modulating microbiome in order to preserve its mutualistic configuration along life span are suggested.

Candela M, Turroni S, Biagi E, Carbonero F, Rampelli S, Fiorentini C, Brigidi P. Inflammation and colorectal cancer, when microbiota-host mutualism breaks. *World J Gastroenterol* 2014; 20(4): 908-922 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/908.htm DOI: http://dx.doi.

org/10.3748/wjg.v20.i4.908

HUMAN INTESTINAL MICROBIOTA

Structure of the human intestinal microbiota

Outnumbering human cells 10 to 1, over 100 trillion microbes are hosted in the human body, with the majority of them residing in the gut, in a continuum of dynamic ecological communities, referred to as microbiome[1]. From 10^1 to 10^3 microbes per gram of content in the stomach and duodenum, the human gut microbiota reaches a microbial density of 10^4 to 10^7 cells per gram in the jejunum and ileum, culminating with 10^{13}-10^{14} cells in the colon and feces[2,3].

Metagenomic surveys of the intestinal microbiota revealed an immense phylogenetic diversity, estimating more than 1000 species-level phylotypes across the human population, with at least 160 prevalent species per individual[4]. While phylogenetic diversity is high at the species level, most of the endogenous bacteria in healthy adults belong to just two phyla, *Firmicutes* and *Bacteroidetes*, which account for > 90% of the known phylogenetic categories of the human gut. Members of *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, *Verrucomicrobia*, *Spirochaetes* and *Lentisphaerae* are regularly present but scarce (< 1%-15%)[5-9].

Since the first application of culture-independent methods a large inter-individual variability in microbial compositions was apparent[4], with twins sharing less than 50% of their species-level microbial taxa[10]. The multiple genetic and environmental factors that contribute to shape the individuality of the gut microbiota composition are now beginning to be understood, reflecting interpersonal, geographical, lifestyle and temporal differences[11-13], and not least, perturbations caused by disease. Recent work has established that despite the unique fingerprint of microbial taxa per individual, a core of > 50 taxa can be found in nearly half of the human subjects sampled[4,8]. It has been suggested that individuals can be categorized into one of three predominant variants or "enterotypes" based on the abundance of dominant genera (*Bacteroides*, *Prevotella* or *Ruminococcus*)[14], though some researchers are now favoring the concept of a continuum or gradient of species functionality rather than a discontinuous variation with segregated types[15]. Individuals have also been shown to share a set of microbial genes involved in central metabolic pathways, and deviations from this functional core have been associated with altered physiological states[16]. However, the subject-specific genetic diversity is remarkable and still remains largely unassigned, with a probably unique metagenomic genotype per individual[17].

Human gut microbiome and role in host physiology

The collective genome of the human intestinal microbiota-microbiome-contains 3.3 million microbial genes, 150-fold more than the human genome[4]. Adding this immense gene catalogue to host genetics, intestinal microorganisms are expected to exert a profound influence on human physiology and metabolism. In fact, gut microbes complement several gaps in our metabolic pathways, *e.g.*, producing essential vitamins and oligoelements, as well as affording the extraction of energy from otherwise indigestible carbohydrates[18], playing a major role in host energy balance and nutrition[19]. This function has probably been the initial evolutionary force toward the microbiota establishment as an animal and human symbiotic partner[20]. Other recognized functions include the support for colonization resistance against incoming enteropathogens. Mechanism involved in this barrier effect are: competition for food resources[21], inhibition of pathogen growth by means of acetate production[22], killing with bacteriocins[23], and immune response stimulation[24,25]. The gut microbiota also acts as an integral component of the human immune system, finely calibrating the immunological potential and responses at different host ages[26,27]. The intimate interplay between gut microbes and the mucosal immune system has indeed proved to be crucial for immune education during our infancy as well as for maintaining a well-balanced immune homeostasis along the adult life[26,28]. Of note, accumulating data are also supporting the emerging concept of a microbiota-gut-brain axis with a role in the regulation of anxiety, cognition, pain and behavior, and a possible contribution to the pathophysiology of central nervous system disorders[29-32].

Microbiota dynamics in response to diet-inflammation-age

The intestinal microbiota composition was believed to be stable throughout adulthood until few years ago[33,34]. More recently, with the bloom of longitudinal studies in humans, the plasticity of this ecosystem has become evident, highlighting that diet, environment, and physiological changes can impact on both composition and functionality of the gut microbiota[12,27]. Faith *et al*[35] investigated the normal long-term plasticity of the human gut microbiota in healthy subjects. By applying a low-error amplicon sequencing approach, the Authors demonstrated that 40% of the individual microbiota was variable over the time course of 5 years.

The effect of changes in dietary habits is the plainest manifestation of the ability of the microbiota to adapt its architecture in response to environmental stimuli, with the speed and efficacy required for the maintenance of the nutritional function of the host-microbiota symbiosis. Indeed, short-term dietary responses of the microbiota composition were detected after 24 h and seemed to be driven principally by the type of ingested fermentable carbohydrates[36,37]. These fluctuations could be considered a necessary feature of an intestinal microbial ecosystem able to rapidly adapt itself to the host requirements, maximizing the efficiency of nutrient extraction and supporting health. Remarkably, the same changes in diet in different persons did not result in the same final microbiota configuration since the diet-related variations did not overcome the inter-individual differences. Conversely,

in the long term, people with similar dietary patterns may end up sharing a similar architecture of the gut microbiota; in fact, it has been shown that the presence or absence of several bacterial taxa can be associated with the intake of different nutrients[37].

Along with the dietary influence, a certain plasticity of the human intestinal ecosystem is being observed in response to less obvious environmental stressors, such as climate and geography[38,39], as well as the degree of exposure to environmental bacteria, the latter being of primary importance for the education and the maintenance of the functionality of the immune system from birth to adulthood[40-42]. Moreover, the consumption of drugs, especially antibiotics but also anti-inflammatory medicines, impacts on the gut microbiota composition[43-45] and different configurations of the microbiota, in turn, have the ability to promote or reduce the metabolization and effectiveness of drugs[46]. Along adulthood and later in life, natural physiological changes add themselves to the list of drivers of modification in the microbiota structure, both temporarily (*i.e.*, pregnancy or lactation[47]) and permanently, as in the aging process.

Aging can impact on the gut microbiota structure directly, by means of age-related physiological processes involving local and systemic inflammation (*i.e.*, immunosenescence and inflamm-aging; see below), and indirectly, causing changes in dietary habits and lifestyle[48]. Increased threshold for taste and smell, together with chewing problems caused by teeth and muscle loss, can lead to the consumption of a restricted diet, poor in fibers and proteins that are known to strongly impact on microbiota composition[36,37]. Moreover, poor diet and diminished physical activity contribute to increase the chances of constipation and, consequently, of slower intestinal transit time, which may impact on the composition of the colonic microbiota due to the reduced bacterial excretion[48]. The age-related increased drug consumption[49] and the interaction between different medicines can also be listed among the possible factors that rule changes in the gut microbiota. The subject-specific combination of all these impacting environmental variables may be responsible for the inter-individual variability of the gut microbiota composition that is known to increase along with aging[50,51].

The aged-type gut microbiota is typically characterized by a reduced biodiversity, an increased abundance of opportunistic facultative anaerobes, and a decreased abundance of species with anti-inflammatory properties (*i.e.*, *Faecalibacterium prausnitzii* and other butyrate producers)[7,44,52-55]. Interestingly, these deviations from the healthy adult-like profile overlap with those known to accompany several disorders characterized by systemic and/or chronic inflammation, such as obesity, metabolic syndrome and inflammatory bowel diseases[21,56,57]. Indeed, aging itself involves chronic immune and inflammatory unbalances. Elderly are generally affected by a process called "immunosenescence" that causes a decline in immune system functionality, and a chronic inflammatory status ("inflamm-aging") characterizing the whole organism[58,59]. At the level of the gut, inflamm-aging could be responsible for an increased stimulation of the inflammatory response, allowing opportunistic pathogens (pathobionts) to thrive to the detriment of symbionts[60,61]. The age-related proliferation of opportunistic bacteria could both contribute to and be nurtured by inflamm-aging, in a sort of self-sustaining loop[55], possibly creating a predisposing environment for diseases the risk of which is known to increase along with age, such as colorectal cancer.

INTESTINAL MICROBIOTA AND COLORECTAL CANCER

Colorectal cancer (CRC) is the fourth most commonly diagnosed cancer in the Western world[62,63]. With more than one million of new cases and 600000 deaths per year, CRC undoubtedly constitutes a significant burden for public health in Western world.

CRC is the result of a multistep process whose progression is associated with the gradual accumulation of genetic and epigenetic mutations. Sporadic in more than 90% of the cases, CRC develops gradually, proceeding from normal epithelium to adenomatous polyps and invasive carcinoma, defining a process that can be slow, taking more than 10 years depending on the mutation frequency[64]. Several genetic predispositions which can increase cancer risk have been identified. The principal driver mutations involved in CRC include tumor suppressors adenomatous polyposis coli gene, β-catenin gene, deleted in colorectal cancer gene and *p53*[64], as well as the oncogenes Kirsten rat sarcoma[65] and myelocytomatosis oncogene[66,67]. However, even if within the last years a growing number of acquired genetic mutations have been described in CRC, trigger factors leading to their accumulation remain to be determined.

Environmental factors have been reported as the leading causes involved in CRC onset[68]. Chronic inflammation and diet have been historically recognized as the prominent CRC drivers[69,70], however, recently, a new potential factor in CRC is emerging: the human intestinal microbiota[71-73]. For instance, the relevance of a compromised microbiota-host homeostasis in CRC onset has been highlighted by the recent finding that mice defective in the inflammasome function have an increased risk to develop CRC[74]. While the involvement of diet and inflammation in CRC has been proved by "traditional" observational and epidemiological studies[70,75-77], only the recent widespread of next-generation sequencing (NGS)-based approaches for gut microbiota characterization allowed to identify characteristic ecosystem changes associated with CRC. Comparative NGS studies of the gut microbiota structure in stools, luminal samples and swabs from CRC patients and age-matched healthy controls have been carried out[78-80]. With respect to healthy controls, CRC patients were significantly enriched in fecal *Fusobacterium*, *Enterococcaceae*, *Campylobacter*, *Erysipelotrichaceae*, *Collinsella*, *Peptostreptococcus* and *Anaerotruncus*, and depleted in members of the *Clostridium* cluster IV, such as *Faecalibacterium prausnitzii* (*F. prausnitzii*) and *Rose-*

buria. On the intestinal mucosa, CRC patients showed an increase of *Porphyromonas*, *Fusobacterium*, *Peptostreptococcus* and *Mogibacterium*, whereas *Faecalibacterium*, *Blautia* and *Bifidobacterium* were depleted. This CRC-associated microbiome is enriched in pro-inflammatory opportunistic pathogens, *e.g.*, *Fusobacterium*, *Enterococcaceae* and *Campylobacter*[81-85], and microorganisms commonly associated with metabolic disorders, such as *Erysipelotrichaceae*[86,87], while depleted in microbial partners strategic to preserve the intestinal homeostasis[88], such as well-known butyrate producers (*i.e.*, *F. prausnitzii* and *Roseburia*) and protective bifidobacteria[22,89]. These NGS data reflect an overall pro-inflammatory configuration for the CRC-associated gut microbial ecosystem, which can concur in compromising the microbiota-host mutualism and, eventually, consolidate the disease state. Very recently, comparative analyses of mucosal microorganisms on cancerous tissue and matched non-cancerous tissue have been carried out, allowing to detect microorganisms specifically enriched on CRC tumor sites[79,84,85]. Cancerous mucosa showed an overall decrease in bacterial diversity with respect to non-cancerous tissues, and was characterized by a reduction in *Faecalibacterium* and higher abundances of *Fusobacterium*, *Bacilli* and *Phascolarctobacterium*. These pro-inflammatory microorganisms can modulate the tumor microenvironment, affecting the course of CRC progression.

In order to explore dysbiosis of the gut microbiota in CRC at the community level, we sought associations between individual genera. To this aim, we obtained co-abundance groups (CAGs), groups of microorganisms which correlate and cluster together, by a bioinformatics analysis[90] of the publicly available dataset from Wu *et al*[80], a well characterized case-control study of the CRC-associated microbiome. Six CAGs displaying significantly different inter-relationships from each other ($P < 0.001$) have been identified: *Fusobacterium* CAG, *Prevotella* CAG, *Barnesiella* CAG, *Coprobacillus* CAG, *Faecalibacterium* CAG and *Bifidobacterium* CAG. Significant associations between bacterial genera have been calculated and represented in a Wiggum plot (Figure 1). This network analysis allowed us to describe - to our knowledge for the first time - microbial co-abundance networks which include microorganisms previously associated with CRC risk or protection. According to our analysis, the CRC-associated microorganisms *Fusobacterium* and *Erysipelotrichaceae* belong to the same CAG (*Fusobacterium*). Analogously, CRC-associated groups as *Enterobacteriaceae*, *Escherichia*, *Shigella* and *Klebsiella* co-vary within the same cluster (*Prevotella*). On the other hand, a common CAG (*Bifidobacterium*) is shared by non-CRC-associated groups as *Bifidobacterium* and *Lachnospiraceae* (a family member of the *Clostridium* cluster IV). Other health-promoting mutualists belonging to the *Clostridium* cluster IV, such as *Faecalibacterim*, *Blautia*, *Roseburia*, *Dorea* and *Lachnospiraceae*, group together in *Faecalibacterim* CAG. Finally, we identified one CAG (*Barnesiella*) including both pro-carcinogenic microorganisms as *Porphyromonadaceae* and *Eubacterium*, as well as protective members of the *Clostridium* cluster IV (*Ruminococcus*, *Butyrococcus* and *Oscillibacter*). Even if data from this computational analysis must be taken with caution since based on a limited dataset, we can hypothesize the existence of 3 pro-carcinogenic CAGs (*Fusobacterium* CAG, *Prevotella* CAG and *Coprobacillus* CAG) and 2 CRC protective CAGs (*Bifidobacterium* CAG and *Faecalibacterium* CAG).

Suggesting the involvement of specific microbiota dysbiosis in CRC, NGS-based microbiome studies are imposing a more holistic vision of the interplay between environment and genetics in CRC, where dietary factors and inflammation need to be considered against the background in the microbiota-host interaction process (Figure 2). However, the static nature of these studies did not permit to comprehend whether dysbiosis are a cause or a consequence of the disease onset. Further, these descriptive studies did not provide information on either the mechanisms by which members of the gut microbial ecosystem can influence the CRC, or, more importantly, the triggers that shift the microbiota towards a carcinogenic configuration. With the attempt to deal with these questions, a new approach to study the role of microorganisms in CRC onset is emerging. Pairing NGS-based microbiota surveys and the usage of germ-free (GF), conventionalized and mono associated mice to test mechanistic hypotheses, new insights on the microbial ecology of CRC have been provided[73].

Bacterial driver-passenger model

Recently, a first dynamic model of the microbial ecology involved in CRC onset and progression has been proposed by Tjalsma *et al*[73]: the bacterial driver-passenger model. According to this model, CRC development is initiated by indigenous bacteria with pro-carcinogenic features - defined as bacterial drivers - that drive epithelial DNA damage and contribute to CRC initiation. In a subsequent step, the local microenvironment is altered as a consequence of the ongoing tumorigenesis and bacterial drivers are replaced by bacterial passengers, microorganisms showing a competitive advantage in the tumor microenvironment and being capable of nurturing tumor progression. For instance, nutrients and co-factors specific of the tumor microenvironment - such as the presence of reactive oxygen species - can be selectively utilized by specific bacterial passengers[91].

Bacterial drivers are defined as intestinal bacteria showing pro-carcinogenic features - either transient or autochthonous microbiota components - that may initiate the process of carcinogenesis. Several candidate bacterial drivers have been identified (Table 1), such as superoxide-producing strains of *Enterococcus faecalis*[92], genotoxin-producing *Escherichia coli* strains[93], and toxigenic strains of *Bacteroides fragilis*[94]. Furthermore, pro-inflammatory members of *Enterobacteriaceae*, such as *Shigella*, *Citrobacter* and *Salmonella* have been associated with early stages of CRC as possible bacterial drivers[95,96]. Occasionally, bacterial drivers act in concert with helper bacteria (or α-bugs) in carcinogenesis promotion[97]. Generally belonging to pro-inflammatory *Enterobacteriaceae*, these microorganisms are proposed to crowd out symbiont CRC-protecting anti-inflammatory microbiota components, such as *F.*

Table 1 Microorganisms involved in colorectal cancer

Microorganism	Role in CRC	Mechanism	Ref.
E. faecalis	Driver	Production of superoxide	[92]
E. coli NC101	Driver	Genotoxin production (colibactin)	[122]
B. fragilis	Driver	Genotoxin production (fragilisin)	[94]
Shigella	Driver	Induction of inflammation	[73]
Citrobacter	Driver	Induction of inflammation	[73]
Salmonella	Driver	Induction of inflammation	[73]
Enterobacteriaceae	Helper	Induction of inflammation	[73]
Fusobacterium	Passenger	Induction of inflammation	[84]
S. gallolyticus	Passenger	Induction of inflammation	[98]
C. septicum	Passenger	Induction of inflammation	[99]
F. prausnitzii	Protective	Butyrate production; anti-inflammatory properties	[78]
Roseburia	Protective	Butyrate production; anti-inflammatory properties	[78]
Bifidobacterium	Protective	Protection from pathogens; anti-inflammatory properties	[71]
Corynebacteriaceae	Protective	Anti-inflammatory properties	[78]

Microorganisms involved in colorectal cancer (CRC), their role as driver, passenger or protective bacteria and the mechanisms involved in CRC induction or protection. *E. faecalis*: *Enterococcus faecalis*; *E. coli*: *Escherichia coli*; *B. fragilis*: *Bacteroides fragilis*; *S. gallolyticus*: *Streptococcus gallolyticus*; *C. septicum*: *Clostridium septicum*; *F. prausnitzii*: *Faecalibacterium prausnitzii*.

prausnitzii, *Roseburia* or *Bifidobacterium*, favoring the subsequent tissue colonization by drivers.

Passenger bacteria are always autochthonous members of the gut microbial community. Relatively poor colonizer of a healthy intestinal tract, passengers show a competitive advantage in the tumor microenvironment (Table 1). However, differently from drivers, which are always pro-carcinogenic, passenger bacteria can be of either pro-carcinogenic or protective nature, depending on the microorganism. While in some cases the carcinogenic tissue has been shown to be selectively colonized by opportunistic pathogens, such as *Fusobacterium*[78,83,84], *Streptococcus gallolyticus*[98] and *Clostridium septicum*[99], which can be involved in CRC progression, in other circumstances the tumor sites were enriched in passenger bacteria belonging to well-known mutualistic microbiota components, as *Corynebacteriaceae*, *Roseburia* and *Faecalibacterium*, suggesting a possible protective role for these microorganisms as CRC quencher[78].

Mechanisms possibly involved in microbial CRC promotion

Gut microorganisms may promote CRC onset and progression by different processes (Table 1)[71], such as (1) the induction of a chronic inflammatory state; (2) the biosynthesis of genotoxins interfering with the cell cycle regulation or directly damaging DNA; (3) the production of toxic metabolites; and (4) the activation of dietary heterocyclic amines to pro-carcinogenic compounds. Here we will specifically discuss the role of three of these factors - inflammation, genotoxins and toxic metabolites – in CRC onset and progression.

Chronic inflammatory disorders are associated with a higher risk of cancer development[100]. Inflammation can nurture carcinogenesis by inducing gene mutations, inhibiting apoptosis or stimulating angiogenesis and cell proliferation. By regulating cell survival, inflammation and immunity, nuclear factor (NF)-κB is at the connection between inflammation and cancer. In particular, experiments carried out in mouse models of colitis-associated cancer have been successful in demonstrating a dual role for NF-κB in carcinogenesis, which depends on the cell type[101]. While in enterocytes NF-κB contributes to tumor initiation by suppressing apoptosis, in myeloid cells it is involved in the promotion of tumor growth by means of the production of inflammatory mediators. Further, it has been recently demonstrated that elevated NF-κB signaling can activate mutations in the Wnt pathway, leading to the differentiation of epithelial non-stem cells into tumor-initiating cells[102]. Generally, the activation of NF-kB results in the expression of inflammatory cytokines [*e.g.*, tumour necrosis factor-alpha, interleukin (IL)-1, IL-6 and IL-8), adhesion molecules, enzymes involved in prostaglandin synthesis, nitric oxide synthase, angiogenic factors and anti-apoptotic genes, providing survival advantages to precancerous or tumor cells in the gut[75,103]. The activation of NF-κB as a result of microbial sensing *via* the host Toll-like receptors (TLRs) has been proposed to support intestinal tumor growth under steady-state conditions[104,105]. Several evidences have been reported in support of the role of the gut microbiota in the inflammation-dependent carcinogenesis in the gut. Crohn's disease and ulcerative colitis are often associated with an increased risk of developing CRC and epidemiological data suggest that duration and severity of chronic colitis represent a significant risk factor for colitis-associated CRC[106,107]. Furthermore, microbiota unbalances in favor of pro-inflammatory opportunistic pathogens as *Enterobacteriaceae* and *Clostridium difficile* have been indicated to be involved in tumor progression[108,109] and, in the context of the bacterial driver-passenger model, several bacterial drivers, such as *Shigella*, *Citrobacter*, *Salmonella* and toxigenic *Bacteroides fragilis* (*B. fragilis*), as well as the passengers *Fusobacterium* and *Streptococcus gallolyticus* and *Clostridium septicum*, have been reported to support carcinogenesis by the induction of a pro-inflammatory response[73]. Strikingly, by inducing azoxymethane (AOM)-colitis in conventional and GF IL-10 knockout (*Il10*$^{-/-}$) mice, Uronis *et al*[110] were

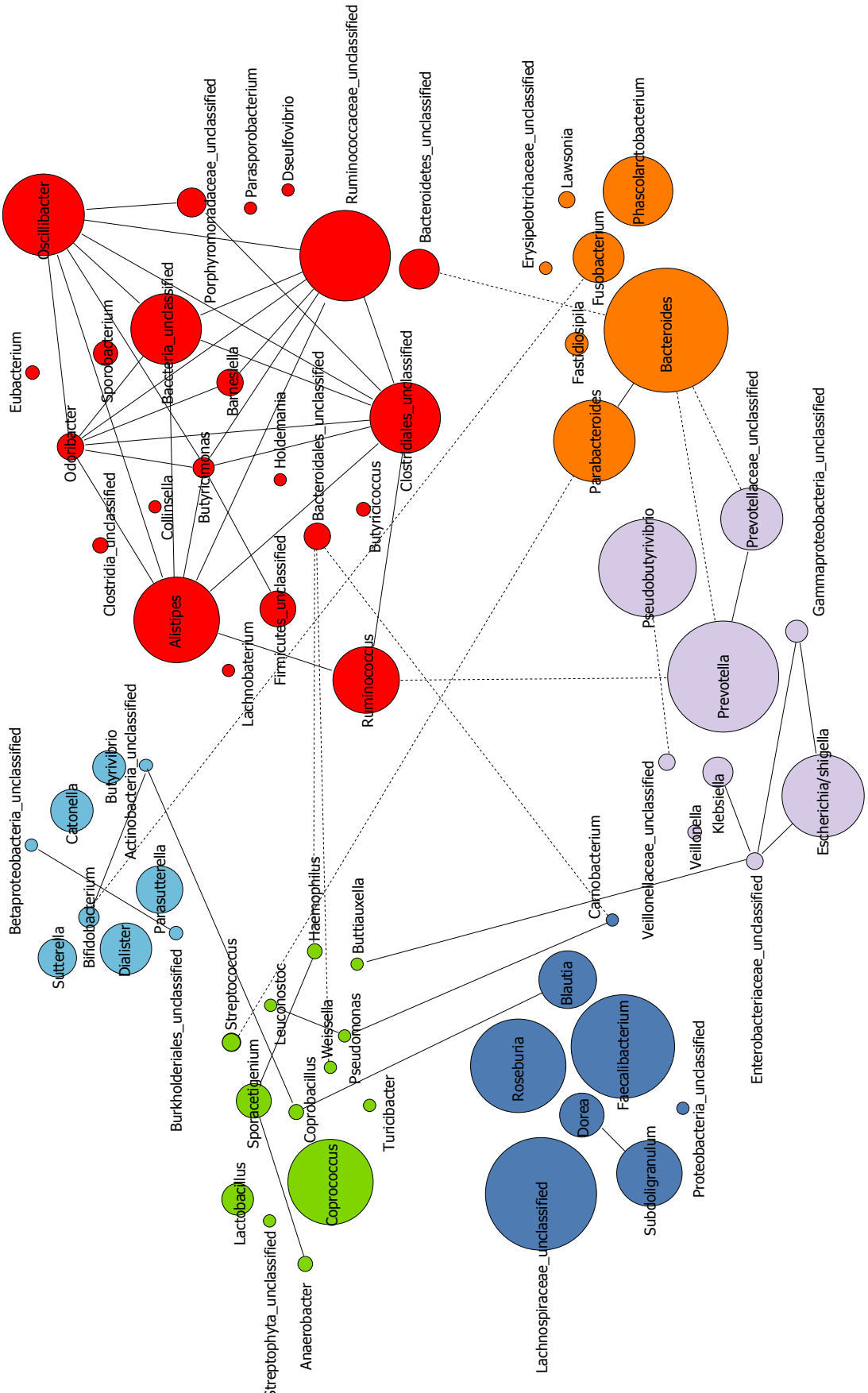


Figure 1 Network plot showing correlation relationships among clusters of bacterial genera for the Wu *et al*[80] dataset. Each node represents a taxon color-coded for co-abundance group and each line highlights a significant correlation between two bacterial genera. Circle size is proportional to genus abundance. Solid lines indicate positive correlation, whereas dot lines indicate negative correlation. Thickness of the lines is proportional to correlation strength.

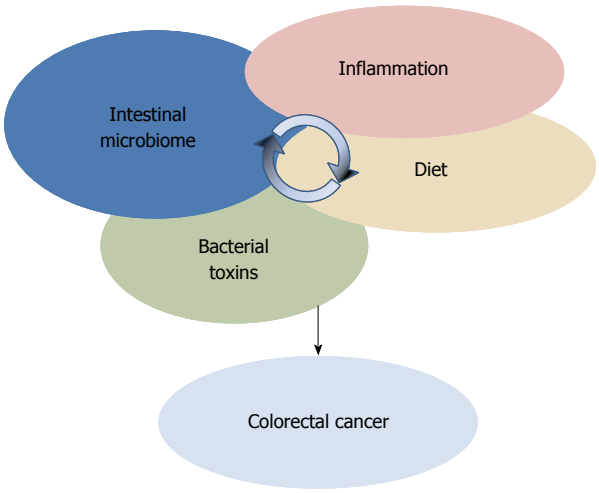


Figure 2 Colorectal cancer arises from the interplay between endogenous and exogenous factors, such as inflammation, diet, intestinal microbiome structure, and transcription and activity of bacterial genotoxins.

successful in demonstrating that microbial sensing *via* TLRs is essential to develop colitis-associated CRC.

Inflammation also represents a molecular link between host immune response, intestinal microbiota and genotoxic events in the inflammation-associated CRC[111]. Several bacterial taxa that belong to the human gut microbiome in a subset of the healthy population contain toxin-producing strains[5]. The long-term effects of chronic exposure to low doses of such bacteria as well as the eventual contribution to the carcinogenic process of bacterial toxins remain to be elucidated. Toxins impinge on key eukaryotic processes, such as cellular signaling, and some directly attack the genome[112] these last by damaging DNA, either directly, by enzymatic attack, or indirectly, by provoking an inflammatory reaction that produces free radicals. Also, they can affect DNA repair mechanisms.

The capacity of the *B. fragilis* toxin (BFT)-producing strains to promote colon tumorigenesis is mediated by the increased expression of STAT3 that leads to the recruitment of the highly pro-inflammatory subset of T helper type 17 lymphocytes, suggesting that the pro-carcinogenic role of BFT is to promote a de-regulated inflammatory response[113]. BFT is a metalloprotease known to bind to colonic epithelial cells and stimulate cleavage of E-cadherin, thus increasing intestinal barrier permeability and augmenting cell signalling *via* the β-catenin/Wnt pathway, which is constitutively activated in essentially all CRC. As a result, BFT stimulates proliferation and migration of human colon cancer cells *in vitro*[114]. It is worth noting that the enterotoxigenic form of *B. fragilis* (ETBF) is only present in approximately 10%-20% of the healthy population whereas the fecal carriage of ETBF is increased of about 40% in CRC patients[94,115].

However, although the *B. fragilis* toxin has been proposed as one of the main CRC driving suspects on the basis of experimental work[113,116], very recent studies show that the most actively transcribed toxins in tumor tissue and surrounding mucosa from CRC patients are those derived from *Escherichia coli* (*E. coli*), *Salmonella enterica* and *Shigella flexneri*. This suggests a strong involvement of enterobacterial toxins in tumorigenesis. Also in this context, inflammation has been shown to increase toxigenic *E. coli* strains, promoting their adhesion to the host epithelia[111]. A number of *E. coli* strains produce a wide array of toxins, some of which are turning out to be potentially harmful in humans, either directly damaging DNA or specifically disrupting cell signaling.

The cytolethal distending toxins (CDTs), which comprise a family of intracellular-acting bacterial protein toxins produced by several gram-negative bacteria, belong to the first group. Their activity upon eukaryotic cells results in several consequences, the most characteristic of which is the induction of G(2)/M cell cycle arrest[117]. Active CDTs consist of three subunits: CdtA and CdtC, which guide internalization, and CdtB, which enzymatically induces DNA double-strand breaks that recruit and activate the ataxia telangiectasia mutated kinase, thus triggering a DNA damage response (DDR). The DDR provides an efficient barrier to tumorigenesis through induction of cell death or senescence[118]. Cells exposed to sub-lethal doses of the CDTs from *Helicobacter hepaticus* (*H. hepaticus*) or *Haemophilus ducreyi* exhibit increased frequency of mutations, accumulation of chromosomal aberrations and enhanced anchorage-independent growth[119]. Furthermore, chronic infection of mouse liver and intestine with CDT-producing *H. hepaticus* or *Campylobacter jejuni*, respectively, is associated with dysplasia[119], confirming the capacity of CDT-producing bacteria to induce preneoplastic lesions *in vivo*. Very recently, Buc *et al*[120] demonstrated a high prevalence of genotoxin- and cyclomodulin-producing mucosa-associated *E. coli* strains in CRC patients.

Furthermore, some commensal *E. coli* strains of the phylogenetic group B2 harbour a 54 kb polyketide synthase (pks) pathogenicity island encoding the enzymes required for the synthesis of a putative hybrid peptide-polyketide genotoxin, named colibactin[121]. Infection of mice with a pks+ *E. coli* strain has been linked to the expression of pks genes required for colibactin production as well as to DNA damage induction[122]. The capacity of colibactin to promote tumorigenesis *in vivo* has been recently proven in an animal model of colitis-associated CRC. GF IL-10 knockout mice treated with the colon-specific carcinogen AOM and monocolonized with pks+ *E. coli* showed a high incidence of invasive adenocarcinoma if compared to mice infected with an isogenic pks-deficient strain or the control commensal bacterium pks-*E. faecalis*[93]. The detection of *E. coli* isolates carrying the pks island in 66.7% CRC patients compared to 20% found in non-inflammatory bowel disease/non-CRC controls suggests a concerted action of host inflammation and *E. coli*-derived pks in giving rise to a host microenvironment that promotes DNA damage and tumorigenesis[93]. These authors also showed that optimal colonization by colibactin-producing *E. coli* strains is established in an

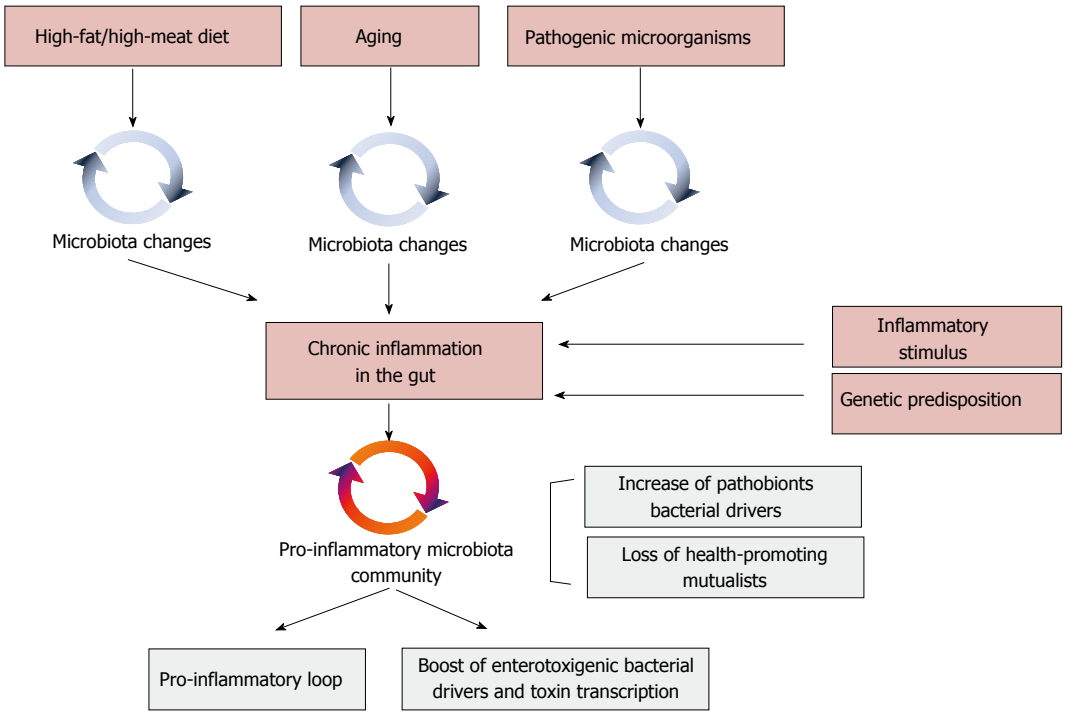


Figure 3 Environmental triggers, such as diet, aging and pathogen infections, can force microbiota changings that, in a genetically susceptible host, can drive to chronic inflammation in the gut. Inflammation shifts the gut microbiota towards a pro-inflammatory configuration, supporting colorectal cancer (CRC) drivers as pathobionts at the expense of health-promoting CRC-protective microbiota components. As a consequence, a pro-inflammatory loop is established in the gut, directly supporting CRC onset and favoring colonization by toxigenic bacterial drivers directly involved in CRC promotion.

already-inflamed gut. In fact, by remodeling the intestinal immune response and shifting the colonic bacterial community to one that further promotes CRC, bacterial drivers permit the colonization of colibactin-producing *E. coli* strains that actively contribute to disease progression.

A second group of toxins includes those disrupting the cell signaling that regulates cell proliferation or induces inflammation. The *E. coli* cytotoxic necrotizing factor 1 (CNF1), which is expressed by many human isolates, activates the Rho GTPases[123], inducing dysfunctions in already transformed epithelial cells, such as apoptosis counteraction, pro-inflammatory cytokines' release, COX2 expression, NF-κB activation and boosted cellular motility. Also, CNF1 induces quiescent cells to enter the cell cycle and undergo DNA synthesis[124], interferes with normal cytokinesis, resulting in the production of multinucleated cells and in the onset of aneuploidia. As cancer may arise when the same regulatory pathways are affected, it is conceivable that CNF1-producing *E. coli* infections can contribute to cancer development[125]. Our hypothesis is that these bacteria may act as passengers, reinforcing and favoring but not causing the development of colorectal cancer. The pro-inflammatory capacity of CNF1 has recently been confirmed in *Drosophila*, where the toxin could activate one of the key transcription factors of the innate immune response, namely NF-κB, independently of the triggering of pathogen recognition receptors. Indeed, the CNF1-mediated activation of the Rac2 GTPase triggers protective immunity *via* the innate Rip kinase signaling that functions upstream of NF-κB[126]. Taken altogether, these data support the strategic role of toxigenic *E. coli* strains in CRC onset and progression.

The gut microbiome is a major driver in shaping the gut metabolome[127]. Among microbial metabolites, several have been identified as potentially important carcinogens or protective. Secondary bile acids in particular have been detected in elevated levels in CRC patient stools and have been shown to have carcinogenic properties *in vitro*[128]. A long list of other metabolites are suspected at varying degrees to be implicated in CRC development, such as hydrogen sulfide[129], proteolysis products (ammonia, amines, phenols)[130], and acetaldehyde[131]. Butyrate is the most sought-after beneficial metabolite as it is a major energy source for colonocytes and more importantly has an anti-proliferative activity and induces apoptosis of CRC cells *in vitro*[132].

Triggering factors that force microbiota to become carcinogenic

Besides its role in CRC onset, inflammation surely exerts a central role in triggering the carcinogenic potential of the gut microbial ecosystem (Figure 3)[93]. Experiments relying on mice defective in components of the immune system successfully demonstrated that chronic inflammation alters the intestinal microbial community composition towards a configuration that predisposes to the disease[133]. According to Garrett *et al*[134], *Tbet-/-/Rag2-/-* mice, which are deficient in adaptive and innate immune function, developed a colitis phenotype transmissible

to wild-type mice by the adoptive transfer of their gut microbiota. Analogously, mice lacking the bacterial flagellin receptor TLR5 exhibited a syndrome encompassing insulin resistance, hyperlipidemia, and increased fat deposition associated with microbiota alterations. Strikingly, these metabolic changes were transferable to wild-type mice by acquiring the *Tlr5*-/- gut microbiota[134]. In this context, Arthur *et al*[93] specifically demonstrated that intestinal inflammation can boost the cancer-inducing activity of the gut microbiota. According to the Authors, chronic inflammation in *Il10*$^{-/-}$ mice was sufficient to prompt microbiota shifts, supporting the AOM-induced carcinogenesis. Favoring the adhesion of driver bacteria with genotoxic potential to the colonic mucosa - as well as the overall expansion of pro-inflammatory *Enterobacteriaceae* in the gut - inflammation creates the environment that supports a bacteria-mediated carcinogenesis process. In particular, Arthur *et al*[93] showed that chronic colitis in *Il10*$^{-/-}$ mice was sufficient to favor a dramatic expansion of *E. coli* NC101 on the intestinal mucosa. Harboring a pks pathogenicity island, *E. coli* NC101 codes for the genotoxin colibactin[121] that allows this microorganism to accelerate progression from dysplasia to invasive carcinoma. Inflammation in the gut is also pivotal to initiate a microbiota-dependent pro-inflammatory loop detrimental for host health[71]. An aberrant inflammatory response in the gut can shift the balance between protective mutualists and pathobionts in favor of the latter[135,136]. By inducing a pro-inflammatory loop, these microorganisms can work as bacterial drivers, consolidating the inflammatory state[28] and resulting in a self-sustained pro-inflammatory response that affects the microbial ecology of the human gut, further compromising the microbiota-host mutualism and supporting CRC.

Abnormal dietary inputs can lead to the expansion of pro-inflammatory microbes in the gut[137]. For instance, a diet rich in saturated milk fat has been reported to induce the expansion of *Bilophila wadsworthia*, which may favor carcinogenesis in the gut by promoting pro-inflammatory T_H1[138]. Indeed, high-fat diet impacts on gut microbiome have seen increased interest in the recent years as fat has been linked epidemiologically to intestinal inflammation and diseases. While as expected a high-fat diet modifies the microbiome, the fact that different fat compositions induced different changes in animal models calls for a more controlled dietary intervention in humans. For example, observational data suggested that Western diet (protein- and fat-enriched) and African diet (polysaccharide-enriched) drive strikingly different microbiomes, possibly explaining different CRC rates[13,38,139]. Reciprocal diet exchanges indeed demonstrated that the microbiome and metabolome were rapidly responsive towards respective "beneficial" and "detrimental" states, as well as markers of mucosal proliferation[13].

The process of human aging has a well-documented impact on the gut microbiota structure[48,140], raising the question of whether age-related microbiota dybioses can trigger a microbiota-dependent carcinogenic process in the gut. Showing a pro-inflammatory configuration, the aged-type gut microbial ecosystem can force a microbiota-dependent pro-inflammatory loop in the gut, compromising the microbiota-host mutualism and supporting carcinogenesis. Strengthening this hypothesis, the incidence of CRC has been reported to increase in the elderly; about 50% of the Western population develops colorectal polyps at the age of 70 and 5% of these polyps progress to cancer[141].

The pervasive role of genotoxins in CRC onset and progression, led researchers to investigate triggering factors that govern toxin biosynthesis and activity. Environmental changes in the gut ecosystem, such as changes in pH, in oxygen availability or the presence of a specific metabolite, have been suggested to have a role in the modulation of toxin transcription. Intriguingly, interspecies quorum sensing resulting from microbe/microbe interaction processes has been suggested to play a role in governing bacterial toxin production in the gut[72,142]. Even if research in this field is still in its infancy, recent experimental works demonstrated the strategic role of microbe/microbe, microbe/host and microbe/environment interaction processes in regulating bacterial virulence and toxin activity. In a recent experimental research based on GF and conventional mice, Kamada *et al*[143] demonstrated that changes in dietary substrates can result in a microbiota-dependent regulation of virulence factors. According to the Authors, dietary changes can boost commensals capable to outcompete toxigenic pathogens for food sources, resulting in the down-regulation of virulence genes and eventually pathogen clearance. Further, Marks *et al*[144] demonstrated that interkingdom signaling as a result of the host response to the influenza A virus infection was sufficient to trigger the expression of *Streptococcus pneumoniae* virulence genes, resulting in the transition from commensalism to pathogenicity. Even if *S. pneumoniae* is a common human nasopharyngeal opportunistic bacterium, these findings allow us to hypothesize the existence of analogous processes in the gut ecosystem, resulting in the activation of a virulence phenotype and toxin transcription of enterotoxigenic CRC drivers.

CONCLUSION

The worldwide diffusion of NGS-based microbiota surveys in CRC patients, alongside the utilization of GF, mono associated and humanized mice, led to an increasing perception of the pivotal role exerted by the gut microbiota in CRC onset and progression. Lights on the microbial ecology of the process have been provided, and possible mechanisms involved suggested. This brought the researchers to focus their attention on triggering factors that turn the intestinal microbiota from a mutualistic configuration to a CRC-promoting asset. Inflammation has undoubtedly a central role in this process, being a common outcome shared by different triggering factors, such as diet, aging, microbe-microbe and microbe-host interactions (Figure 3). In fact, changes in diet, aging, as well as pathobiont-dependent pro-inflammatory dysbiosis of the gut microbiota, can force the gut microbiota to

a pro-inflammatory asset, changing the microecology of the gut ecosystem and activating toxigenic CRC bacterial drivers. In this context, of extraordinary importance will be the development of strategies able to interfere and/or block these triggering factors, preserving the microbiota-host mutualism along the entire life span. Different approaches can be implemented. Since diet represents the pivotal strategy to modulate composition and functionality of the gut microbiota, the most promising approach to preserve microbiota-host mutualisms relies on dietary interventions. For instance, diet can be modulated to boost health-promoting microbiota groups, such as anti-inflammatory members of the *Clostridium* cluster IV or short chain fatty acid producers of the *Clostridium* cluster XIVa. Strengthening this perspective, in a life-long longitudinal study carried out in mice, Zhang *et al*[145] demonstrated that different diets modulated differently the microbiome trajectories along with aging. In particular, according to the Authors low-fat diet and caloric restriction increased the relative abundance of phylotypes positively associated with the life span in the middle-life, and, at the same time, lowered the abundance of opportunistic pro-inflammatory pathogens, which could represent CRC bacterial drivers.

A second approach for CRC prevention surely relies on the usage of probiotic bacteria, such as *Bifidobacterium* and *Lactobacillus*. Probiotics have been demonstrated to be effective in reducing CRC risk in humans[146-149]. Showing immunomodulating properties, antimicrobial activities, as well as the capacity to interfere with toxin synthesis and activity, probiotic bacteria can act simultaneously on different CRC triggering factors. In fact, probiotics have been reported as effective in quenching host inflammatory response[150], in inhibiting the colonization of known CRC drivers[22,151] and in inactivating bacterial toxins[152] or interfering with their production[153,154].

Even if significant steps forward have been carried out, we are still far from fully appreciating the multifactorial role of the intestinal microbiota in CRC. More longitudinal microbiome surveys need to be carried out, and intestinal polyps as well as adenocarcinoma tissues must be sampled, in order to follow the gut microbiota dynamics over time for the development of colonic neoplasia. Microbiota on tumor sites needs to be compared with off-tumor matched tissues, as a better comparison than mucosal samples from healthy patients. Associations between structure and dynamics of the gut microbiome and the different stages of colonic neoplasia need to be better defined, causality should be further explored, possibly by using GF, mono associated as well as humanized animal models. Finally, meta-analysis integrating epidemiological studies with microbiome datasets will allow us to better define triggering factors that force the microbiota to become carcinogenic, so that hypotheses can be verified in mice where possible intervention strategies can be tested.

REFERENCES

1 **Turnbaugh PJ**, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. *Nature* 2007; **449**: 804-810 [PMID: 17943116]

2 **Dethlefsen L**, Eckburg PB, Bik EM, Relman DA. Assembly of the human intestinal microbiota. *Trends Ecol Evol* 2006; **21**: 517-523 [PMID: 16820245]

3 **O'Hara AM**, Shanahan F. The gut flora as a forgotten organ. *EMBO Rep* 2006; **7**: 688-693 [PMID: 16819463]

4 **Qin J**, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T, Mende DR, Li J, Xu J, Li S, Li D, Cao J, Wang B, Liang H, Zheng H, Xie Y, Tap J, Lepage P, Bertalan M, Batto JM, Hansen T, Le Paslier D, Linneberg A, Nielsen HB, Pelletier E, Renault P, Sicheritz-Ponten T, Turner K, Zhu H, Yu C, Li S, Jian M, Zhou Y, Li Y, Zhang X, Li S, Qin N, Yang H, Wang J, Brunak S, Doré J, Guarner F, Kristiansen K, Pedersen O, Parkhill J, Weissenbach J, Bork P, Ehrlich SD, Wang J. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* 2010; **464**: 59-65 [PMID: 20203603 DOI: 10.1038/nature08821]

5 **Eckburg PB**, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA. Diversity of the human intestinal microbial flora. *Science* 2005; **308**: 1635-1638 [PMID: 15831718]

6 **Rajilić-Stojanović M**, Smidt H, de Vos WM. Diversity of the human gastrointestinal tract microbiota revisited. *Environ Microbiol* 2007; **9**: 2125-2136 [PMID: 17686012]

7 **Rajilić-Stojanović M**, Heilig HG, Molenaar D, Kajander K, Surakka A, Smidt H, de Vos WM. Development and application of the human intestinal tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. *Environ Microbiol* 2009; **11**: 1736-1751 [PMID: 19508560 DOI: 10.1111/j.1462-2920.2009.01900.x]

8 **Tap J**, Mondot S, Levenez F, Pelletier E, Caron C, Furet JP, Ugarte E, Muñoz-Tamayo R, Paslier DL, Nalin R, Dore J, Leclerc M. Towards the human intestinal microbiota phylogenetic core. *Environ Microbiol* 2009; **11**: 2574-2584 [PMID: 19601958 DOI: 10.1111/j.1462-2920.2009.01982.x]

9 **Human Microbiome Project Consortium.** Structure, function and diversity of the healthy human microbiome. *Nature* 2012; **486**: 207-214 [PMID: 22699609 DOI: 10.1038/nature11234]

10 **Turnbaugh PJ**, Quince C, Faith JJ, McHardy AC, Yatsunenko T, Niazi F, Affourtit J, Egholm M, Henrissat B, Knight R, Gordon JI. Organismal, genetic, and transcriptional variation in the deeply sequenced gut microbiomes of identical twins. *Proc Natl Acad Sci USA* 2010; **107**: 7503-7508 [PMID: 20363958 DOI: 10.1073/pnas.1002355107]

11 **Benson AK**, Kelly SA, Legge R, Ma F, Low SJ, Kim J, Zhang M, Oh PL, Nehrenberg D, Hua K, Kachman SD, Moriyama EN, Walter J, Peterson DA, Pomp D. Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. *Proc Natl Acad Sci USA* 2010; **107**: 18933-18938 [PMID: 20937875 DOI: 10.1073/pnas.1007028107]

12 **Candela M**, Biagi E, Maccaferri S, Turroni S, Brigidi P. Intestinal microbiota is a plastic factor responding to environmental changes. *Trends Microbiol* 2012; **20**: 385-391 [PMID: 22672911 DOI: 10.1016/j.tim.2012.05.003]

13 **Ou J**, Carbonero F, Zoetendal EG, DeLany JP, Wang M, Newton K, Gaskins HR, O'Keefe SJ. Diet, microbiota, and microbial metabolites in colon cancer risk in rural Africans and African Americans. *Am J Clin Nutr* 2013; **98**: 111-120 [PMID: 23719549 DOI: 10.3945/ajcn.112.056689]

14 **Arumugam M**, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto JM, Bertalan M, Borruel N, Casellas F, Fernandez L, Gautier L, Hansen T, Hattori M, Hayashi T, Kleerebezem M, Kurokawa K, Leclerc M, Levenez F, Manichanh C, Nielsen HB, Nielsen T, Pons N, Poulain J, Qin J, Sicheritz-Ponten T, Tims S, Torrents D,

Ugarte E, Zoetendal EG, Wang J, Guarner F, Pedersen O, de Vos WM, Brunak S, Doré J, Antolín M, Artiguenave F, Blottiere HM, Almeida M, Brechot C, Cara C, Chervaux C, Cultrone A, Delorme C, Denariaz G, Dervyn R, Foerstner KU, Friss C, van de Guchte M, Guedon E, Haimet F, Huber W, van Hylckama-Vlieg J, Jamet A, Juste C, Kaci G, Knol J, Lakhdari O, Layec S, Le Roux K, Maguin E, Mérieux A, Melo Minardi R, M'rini C, Muller J, Oozeer R, Parkhill J, Renault P, Rescigno M, Sanchez N, Sunagawa S, Torrejon A, Turner K, Vandemeulebrouck G, Varela E, Winogradsky Y, Zeller G, Weissenbach J, Ehrlich SD, Bork P. Enterotypes of the human gut microbiome. *Nature* 2011; **473**: 174-180 [PMID: 21508958 DOI: 10.1038/nature09944]

15 **Jeffery IB**, Claesson MJ, O'Toole PW, Shanahan F. Categorization of the gut microbiota: enterotypes or gradients? *Nat Rev Microbiol* 2012; **10**: 591-592 [PMID: 23066529]

16 **Turnbaugh PJ**, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, Egholm M, Henrissat B, Heath AC, Knight R, Gordon JI. A core gut microbiome in obese and lean twins. *Nature* 2009; **457**: 480-484 [PMID: 19043404 DOI: 10.1038/nature07540]

17 **Schloissnig S**, Arumugam M, Sunagawa S, Mitreva M, Tap J, Zhu A, Waller A, Mende DR, Kultima JR, Martin J, Kota K, Sunyaev SR, Weinstock GM, Bork P. Genomic variation landscape of the human gut microbiome. *Nature* 2013; **493**: 45-50 [PMID: 23222524 DOI: 10.1038/nature11711]

18 **Koropatkin NM**, Cameron EA, Martens EC. How glycan metabolism shapes the human gut microbiota. *Nat Rev Microbiol* 2012; **10**: 323-335 [PMID: 22491358 DOI: 10.1038/nrmicro2746]

19 **Bäckhed F**, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI. The gut microbiota as an environmental factor that regulates fat storage. *Proc Natl Acad Sci USA* 2004; **101**: 15718-15723 [PMID: 15505215]

20 **Clemente JC**, Ursell LK, Parfrey LW, Knight R. The impact of the gut microbiota on human health: an integrative view. *Cell* 2012; **148**: 1258-1270 [PMID: 22424233 DOI: 10.1016/j.cell.2012.01.035]

21 **Neish AS**. Microbes in gastrointestinal health and disease. *Gastroenterology* 2009; **136**: 65-80 [PMID: 19026645 DOI: 10.1053/j.gastro.2008.10.080]

22 **Fukuda S**, Toh H, Hase K, Oshima K, Nakanishi Y, Yoshimura K, Tobe T, Clarke JM, Topping DL, Suzuki T, Taylor TD, Itoh K, Kikuchi J, Morita H, Hattori M, Ohno H. Bifidobacteria can protect from enteropathogenic infection through production of acetate. *Nature* 2011; **469**: 543-547 [PMID: 21270894 DOI: 10.1038/nature09646]

23 **Corr SC**, Li Y, Riedel CU, O'Toole PW, Hill C, Gahan CG. Bacteriocin production as a mechanism for the antiinfective activity of Lactobacillus salivarius UCC118. *Proc Natl Acad Sci USA* 2007; **104**: 7617-7621 [PMID: 17456596]

24 **Keeney KM**, Finlay BB. Enteric pathogen exploitation of the microbiota-generated nutrient environment of the gut. *Curr Opin Microbiol* 2011; **14**: 92-98 [PMID: 21215681 DOI: 10.1016/j.mib.2010.12.012]

25 **Stecher B**, Hardt WD. Mechanisms controlling pathogen colonization of the gut. *Curr Opin Microbiol* 2011; **14**: 82-91 [PMID: 21036098 DOI: 10.1016/j.mib.2010.10.003]

26 **Maynard CL**, Elson CO, Hatton RD, Weaver CT. Reciprocal interactions of the intestinal microbiota and immune system. *Nature* 2012; **489**: 231-241 [PMID: 22972296 DOI: 10.1038/nature11551]

27 **Candela M**, Biagi E, Turroni S, Maccaferri S, Figini P, Brigidi P. Candela M, Biagi E, Turroni S, Maccaferri S, Figini P, Brigidi P. Dynamic efficiency of the human intestinal microbiota. *Crit Rev Microbiol* 2013; In press

28 **Round JL**, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* 2009; **9**: 313-323 [PMID: 19343057 DOI: 10.1038/nri2515]

29 **Collins SM**, Bercik P. The relationship between intestinal microbiota and the central nervous system in normal gastrointestinal function and disease. *Gastroenterology* 2009; **136**: 2003-2014 [PMID: 19457424 DOI: 10.1053/j.gastro.2009.01.075]

30 **Rhee SH**, Pothoulakis C, Mayer EA. Principles and clinical implications of the brain-gut-enteric microbiota axis. *Nat Rev Gastroenterol Hepatol* 2009; **6**: 306-314 [PMID: 19404271 DOI: 10.1038/nrgastro.2009.35]

31 **Cryan JF**, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci* 2012; **13**: 701-712 [PMID: 22968153 DOI: 10.1038/nrn3346]

32 **Collins SM**, Bercik P. Gut microbiota: Intestinal bacteria influence brain activity in healthy humans. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 326-327 [PMID: 23648940 DOI: 10.1038/nrgastro.2013.76]

33 **Zoetendal EG**, Akkermans AD, De Vos WM. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Appl Environ Microbiol* 1998; **64**: 3854-3859 [PMID: 9758810]

34 **Guarner F**, Malagelada JR. Gut flora in health and disease. *Lancet* 2003; **361**: 512-519 [PMID: 12583961]

35 **Faith JJ**, Guruge JL, Charbonneau M, Subramanian S, Seedorf H, Goodman AL, Clemente JC, Knight R, Heath AC, Leibel RL, Rosenbaum M, Gordon JI. The long-term stability of the human gut microbiota. *Science* 2013; **341**: 1237439 [PMID: 23828941 DOI: 10.1126/science.1237439]

36 **Walker AW**, Ince J, Duncan SH, Webster LM, Holtrop G, Ze X, Brown D, Stares MD, Scott P, Bergerat A, Louis P, McIntosh F, Johnstone AM, Lobley GE, Parkhill J, Flint HJ. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. *ISME J* 2011; **5**: 220-230 [PMID: 20686513 DOI: 10.1038/ismej.2010.118]

37 **Wu GD**, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, Bewtra M, Knights D, Walters WA, Knight R, Sinha R, Gilroy E, Gupta K, Baldassano R, Nessel L, Li H, Bushman FD, Lewis JD. Linking long-term dietary patterns with gut microbial enterotypes. *Science* 2011; **334**: 105-108 [PMID: 21885731 DOI: 10.1126/science.1208344]

38 **De Filippo C**, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G, Lionetti P. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Natl Acad Sci USA* 2010; **107**: 14691-14696 [PMID: 20679230 DOI: 10.1073/pnas.1005963107]

39 **Jalanka-Tuovinen J**, Salonen A, Nikkilä J, Immonen O, Kekkonen R, Lahti L, Palva A, de Vos WM. Intestinal microbiota in healthy adults: temporal analysis reveals individual and common core and relation to intestinal symptoms. *PLoS One* 2011; **6**: e23035 [PMID: 21829582 DOI: 10.1371/journal.pone.0023035]

40 **Blaser MJ**, Falkow S. What are the consequences of the disappearing human microbiota? *Nat Rev Microbiol* 2009; **7**: 887-894 [PMID: 19898491 DOI: 10.1038/nrmicro2245]

41 **Ehlers S**, Kaufmann SH. Infection, inflammation, and chronic diseases: consequences of a modern lifestyle. *Trends Immunol* 2010; **31**: 184-190 [PMID: 20399709 DOI: 10.1016/j.it.2010.02.003]

42 **Ohnmacht C**, Marques R, Presley L, Sawa S, Lochner M, Eberl G. Intestinal microbiota, evolution of the immune system and the bad reputation of pro-inflammatory immunity. *Cell Microbiol* 2011; **13**: 653-659 [PMID: 21338464 DOI: 10.1111/j.1462-5822.2011.01577.x]

43 **Jernberg C**, Löfmark S, Edlund C, Jansson JK. Long-term impacts of antibiotic exposure on the human intestinal microbiota. *Microbiology* 2010; **156**: 3216-3223 [PMID: 20705661 DOI: 10.1099/mic.0.040618-0]

44 **Mäkivuokko H**, Tiihonen K, Tynkkynen S, Paulin L, Rau-

tonen N. The effect of age and non-steroidal anti-inflammatory drugs on human intestinal microbiota composition. *Br J Nutr* 2010; **103**: 227-234 [PMID: 19703328 DOI: 10.1017/S0007114509991553]
45 **Dethlefsen L**, Relman DA. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. *Proc Natl Acad Sci USA* 2011; **108** Suppl 1: 4554-4561 [PMID: 20847294 DOI: 10.1073/pnas.1000087107]
46 **Viswanathan V.** The meddling microbes midst our medicines. *Gut Microbes* 2013; Epub ahead of print [PMID: 23969979]
47 **Koren O**, Goodrich JK, Cullender TC, Spor A, Laitinen K, Bäckhed HK, Gonzalez A, Werner JJ, Angenent LT, Knight R, Bäckhed F, Isolauri E, Salminen S, Ley RE. Host remodeling of the gut microbiome and metabolic changes during pregnancy. *Cell* 2012; **150**: 470-480 [PMID: 22863002 DOI: 10.1016/j.cell.2012.07.008]
48 **Biagi E**, Candela M, Fairweather-Tait S, Franceschi C, Brigidi P. Aging of the human metaorganism: the microbial counterpart. *Age* (Dordr) 2012; **34**: 247-267 [PMID: 21347607 DOI: 10.1007/s11357-011-9217-5]
49 **Genser D**. Food and drug interaction: consequences for the nutrition/health status. *Ann Nutr Metab* 2008; **52** Suppl 1: 29-32 [PMID: 18382075 DOI: 10.1159/000115345]
50 **Claesson MJ**, Cusack S, O'Sullivan O, Greene-Diniz R, de Weerd H, Flannery E, Marchesi JR, Falush D, Dinan T, Fitzgerald G, Stanton C, van Sinderen D, O'Connor M, Harnedy N, O'Connor K, Henry C, O'Mahony D, Fitzgerald AP, Shanahan F, Twomey C, Hill C, Ross RP, O'Toole PW. Composition, variability, and temporal stability of the intestinal microbiota of the elderly. *Proc Natl Acad Sci USA* 2011; **108** Suppl 1: 4586-4591 [PMID: 20571116 DOI: 10.1073/pnas.1000097107]
51 **Rea MC**, O'Sullivan O, Shanahan F, O'Toole PW, Stanton C, Ross RP, Hill C. Clostridium difficile carriage in elderly subjects and associated changes in the intestinal microbiota. *J Clin Microbiol* 2012; **50**: 867-875 [PMID: 22162545 DOI: 10.1128/JCM.05176-11]
52 **Hayashi H**, Sakamoto M, Kitahara M, Benno Y. Molecular analysis of fecal microbiota in elderly individuals using 16S rDNA library and T-RFLP. *Microbiol Immunol* 2003; **47**: 557-570 [PMID: 14524616]
53 **Mueller S**, Saunier K, Hanisch C, Norin E, Alm L, Midtvedt T, Cresci A, Silvi S, Orpianesi C, Verdenelli MC, Clavel T, Koebnick C, Zunft HJ, Doré J, Blaut M. Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. *Appl Environ Microbiol* 2006; **72**: 1027-1033 [PMID: 16461645]
54 **Mariat D**, Firmesse O, Levenez F, Guimarăes V, Sokol H, Doré J, Corthier G, Furet JP. The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. *BMC Microbiol* 2009; **9**: 123 [PMID: 19508720 DOI: 10.1186/1471-2180-9-123]
55 **Biagi E**, Nylund L, Candela M, Ostan R, Bucci L, Pini E, Nikkïla J, Monti D, Satokari R, Franceschi C, Brigidi P, De Vos W. Through ageing, and beyond: gut microbiota and inflammatory status in seniors and centenarians. *PLoS One* 2010; **5**: e10667 [PMID: 20498852 DOI: 10.1371/journal.pone.0010667]
56 **Cani PD**, Delzenne NM. The role of the gut microbiota in energy metabolism and metabolic disease. *Curr Pharm Des* 2009; **15**: 1546-1558 [PMID: 19442172]
57 **Maslowski KM**, Mackay CR. Diet, gut microbiota and immune responses. *Nat Immunol* 2011; **12**: 5-9 [PMID: 21169997 DOI: 10.1038/ni0111-5]
58 **Ostan R**, Bucci L, Capri M, Salvioli S, Scurti M, Pini E, Monti D, Franceschi C. Immunosenescence and immunogenetics of human longevity. *Neuroimmunomodulation* 2008; **15**: 224-240 [PMID: 19047800 DOI: 10.1159/000156466]
59 **Larbi A**, Franceschi C, Mazzatti D, Solana R, Wikby A, Pawelec G. Aging of the immune system as a prognostic factor for human longevity. *Physiology (Bethesda)* 2008; **23**: 64-74 [PMID: 18400689 DOI: 10.1152/physiol.00040.2007]
60 **Guigoz Y**, Doré J, Schiffrin EJ. The inflammatory status of old age can be nurtured from the intestinal environment. *Curr Opin Clin Nutr Metab Care* 2008; **11**: 13-20 [PMID: 18090652]
61 **Sansonetti PJ**. To be or not to be a pathogen: that is the mucosally relevant question. *Mucosal Immunol* 2011; **4**: 8-14 [PMID: 21150896 DOI: 10.1038/mi.2010.77]
62 **Boyle P**, Ferlay J. Cancer incidence and mortality in Europe, 2004. *Ann Oncol* 2005; **16**: 481-488 [PMID: 15718248]
63 **Ferlay J**, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; **127**: 2893-2917 [PMID: 21351269 DOI: 10.1002/ijc.25516]
64 **Vogelstein B**, Kinzler KW. Colorectal tumors. The Genetic Basis of Human Cancer. New York: McGraw-Hill, 1998: 565-587
65 **Lengauer C**, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. *Nature* 1998; **396**: 643-649 [PMID: 9872311]
66 **Sheng H**, Shao J, Williams CS, Pereira MA, Taketo MM, Oshima M, Reynolds AB, Washington MK, DuBois RN, Beauchamp RD. Nuclear translocation of beta-catenin in hereditary and carcinogen-induced intestinal adenomas. *Carcinogenesis* 1998; **19**: 543-549 [PMID: 9600336]
67 **Lakatos PL**, Lakatos L. Risk for colorectal cancer in ulcerative colitis: changes, causes and management strategies. *World J Gastroenterol* 2008; **14**: 3937-3947 [PMID: 18609676]
68 **Lichtenstein P**, Holm NV, Verkasalo PK, Iliadou A, Kaprio J, Koskenvuo M, Pukkala E, Skytthe A, Hemminki K. Environmental and heritable factors in the causation of cancer--analyses of cohorts of twins from Sweden, Denmark, and Finland. *N Engl J Med* 2000; **343**: 78-85 [PMID: 10891514]
69 **Bingham SA**. Diet and colorectal cancer prevention. *Biochem Soc Trans* 2000; **28**: 12-16 [PMID: 10816091]
70 **Hope ME**, Hold GL, Kain R, El-Omar EM. Sporadic colorectal cancer--role of the commensal microbiota. *FEMS Microbiol Lett* 2005; **244**: 1-7 [PMID: 15727814]
71 **Candela M**, Guidotti M, Fabbri A, Brigidi P, Franceschi C, Fiorentini C. Human intestinal microbiota: cross-talk with the host and its potential role in colorectal cancer. *Crit Rev Microbiol* 2011; **37**: 1-14 [PMID: 20874522 DOI: 10.3109/1040841X.2010.501760]
72 **Plottel CS**, Blaser MJ. Microbiome and malignancy. *Cell Host Microbe* 2011; **10**: 324-335 [PMID: 22018233 DOI: 10.1016/j.chom.2011.10.003]
73 **Tjalsma H**, Boleij A, Marchesi JR, Dutilh BE. A bacterial driver-passenger model for colorectal cancer: beyond the usual suspects. *Nat Rev Microbiol* 2012; **10**: 575-582 [PMID: 22728587 DOI: 10.1038/nrmicro2819]
74 **Hirota SA**, Ng J, Lueng A, Khajah M, Parhar K, Li Y, Lam V, Potentier MS, Ng K, Bawa M, McCafferty DM, Rioux KP, Ghosh S, Xavier RJ, Colgan SP, Tschopp J, Muruve D, MacDonald JA, Beck PL. NLRP3 inflammasome plays a key role in the regulation of intestinal homeostasis. *Inflamm Bowel Dis* 2011; **17**: 1359-1372 [PMID: 20872834 DOI: 10.1002/ibd.21478]
75 **Horst D**, Reu S, Kriegl L, Engel J, Kirchner T, Jung A. The intratumoral distribution of nuclear beta-catenin is a prognostic marker in colon cancer. *Cancer* 2009; **115**: 2063-2070 [PMID: 19298008 DOI: 10.1002/cncr.24254]
76 **Mai V**, McCrary QM, Sinha R, Glei M. Associations between dietary habits and body mass index with gut microbiota composition and fecal water genotoxicity: an observational study in African American and Caucasian American volunteers. *Nutr J* 2009; **8**: 49 [PMID: 19845958 DOI: 10.1186/1475-2891-8-49]
77 **McConnell BB**, Yang VW. The Role of Inflammation in the Pathogenesis of Colorectal Cancer. *Curr Colorectal Cancer Rep* 2009; **5**: 69-74 [PMID: 19756239]
78 **Marchesi JR**, Dutilh BE, Hall N, Peters WH, Roelofs R, Boleij A, Tjalsma H. Towards the human colorectal cancer

microbiome. *PLoS One* 2011; **6**: e20447 [PMID: 21647227 DOI: 10.1371/journal.pone.0020447]
79 **Chen W**, Liu F, Ling Z, Tong X, Xiang C. Human intestinal lumen and mucosa-associated microbiota in patients with colorectal cancer. *PLoS One* 2012; **7**: e39743 [PMID: 22761885 DOI: 10.1371/journal.pone.0039743]
80 **Wu N**, Yang X, Zhang R, Li J, Xiao X, Hu Y, Chen Y, Yang F, Lu N, Wang Z, Luan C, Liu Y, Wang B, Xiang C, Wang Y, Zhao F, Gao GF, Wang S, Li L, Zhang H, Zhu B. Dysbiosis signature of fecal microbiota in colorectal cancer patients. *Microb Ecol* 2013; **66**: 462-470 [PMID: 23733170 DOI: 10.1007/s00248-013-0245-9]
81 **Wang X**, Huycke MM. Extracellular superoxide production by Enterococcus faecalis promotes chromosomal instability in mammalian cells. *Gastroenterology* 2007; **132**: 551-561 [PMID: 17258726]
82 **Zheng J**, Meng J, Zhao S, Singh R, Song W. Campylobacter-induced interleukin-8 secretion in polarized human intestinal epithelial cells requires Campylobacter-secreted cytolethal distending toxin- and Toll-like receptor-mediated activation of NF-kappaB. *Infect Immun* 2008; **76**: 4498-4508 [PMID: 18644884 DOI: 10.1128/IAI.01317-07]
83 **Castellarin M**, Warren RL, Freeman JD, Dreolini L, Krzywinski M, Strauss J, Barnes R, Watson P, Allen-Vercoe E, Moore RA, Holt RA. Fusobacterium nucleatum infection is prevalent in human colorectal carcinoma. *Genome Res* 2012; **22**: 299-306 [PMID: 22009989 DOI: 10.1101/gr.126516.111]
84 **Kostic AD**, Chun E, Robertson L, Glickman JN, Gallini CA, Michaud M, Clancy TE, Chung DC, Lochhead P, Hold GL, El-Omar EM, Brenner D, Fuchs CS, Meyerson M, Garrett WS. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. *Cell Host Microbe* 2013; **14**: 207-215 [PMID: 23954159 DOI: 10.1016/j.chom.2013.07.007]
85 **McCoy AN**, Araújo-Pérez F, Azcárate-Peril A, Yeh JJ, Sandler RS, Keku TO. Fusobacterium is associated with colorectal adenomas. *PLoS One* 2013; **8**: e53653 [PMID: 23335968 DOI: 10.1371/journal.pone.0053653]
86 **Turnbaugh PJ**, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. *Sci Transl Med* 2009; **1**: 6ra14 [PMID: 20368178 DOI: 10.1126/scitranslmed.3000322]
87 **Martínez I**, Perdicaro DJ, Brown AW, Hammons S, Carden TJ, Carr TP, Eskridge KM, Walter J. Diet-induced alterations of host cholesterol metabolism are likely to affect the gut microbiota composition in hamsters. *Appl Environ Microbiol* 2013; **79**: 516-524 [PMID: 23124234 DOI: 10.1128/AEM.03046-12]
88 **Louis P**, Flint HJ. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiol Lett* 2009; **294**: 1-8 [PMID: 19222573 DOI: 10.1111/j.1574-6968.2009.01514.x]
89 **Fanning S**, Hall LJ, Cronin M, Zomer A, MacSharry J, Goulding D, Motherway MO, Shanahan F, Nally K, Dougan G, van Sinderen D. Bifidobacterial surface-exopolysaccharide facilitates commensal-host interaction through immune modulation and pathogen protection. *Proc Natl Acad Sci USA* 2012; **109**: 2108-2113 [PMID: 22308390 DOI: 10.1073/pnas.1115621109]
90 **Claesson MJ**, Jeffery IB, Conde S, Power SE, O'Connor EM, Cusack S, Harris HM, Coakley M, Lakshminarayanan B, O'Sullivan O, Fitzgerald GF, Deane J, O'Connor M, Harnedy N, O'Connor K, O'Mahony D, van Sinderen D, Wallace M, Brennan L, Stanton C, Marchesi JR, Fitzgerald AP, Shanahan F, Hill C, Ross RP, O'Toole PW. Gut microbiota composition correlates with diet and health in the elderly. *Nature* 2012; **488**: 178-184 [PMID: 22797518 DOI: 10.1038/nature11319]
91 **Bliska JB**, van der Velden AW. Salmonella "sops" up a preferred electron receptor in the inflamed intestine. *MBio* 2012; **3**: e00226-e00212 [PMID: 22893385 DOI: 10.1128/mBio.00226-12]
92 **Huycke MM**, Abrams V, Moore DR. Enterococcus faecalis produces extracellular superoxide and hydrogen peroxide that damages colonic epithelial cell DNA. *Carcinogenesis* 2002; **23**: 529-536 [PMID: 11895869]
93 **Arthur JC**, Perez-Chanona E, Mühlbauer M, Tomkovich S, Uronis JM, Fan TJ, Campbell BJ, Abujamel T, Dogan B, Rogers AB, Rhodes JM, Stintzi A, Simpson KW, Hansen JJ, Keku TO, Fodor AA, Jobin C. Intestinal inflammation targets cancer-inducing activity of the microbiota. *Science* 2012; **338**: 120-123 [PMID: 22903521 DOI: 10.1126/science.1224820]
94 **Toprak NU**, Yagci A, Gulluoglu BM, Akin ML, Demirkalem P, Celenk T, Soyletir G. A possible role of Bacteroides fragilis enterotoxin in the aetiology of colorectal cancer. *Clin Microbiol Infect* 2006; **12**: 782-786 [PMID: 16842574]
95 **Ahmed S**, Macfarlane GT, Fite A, McBain AJ, Gilbert P, Macfarlane S. Mucosa-associated bacterial diversity in relation to human terminal ileum and colonic biopsy samples. *Appl Environ Microbiol* 2007; **73**: 7435-7442 [PMID: 17890331]
96 **Shen XJ**, Rawls JF, Randall T, Burcal L, Mpande CN, Jenkins N, Jovov B, Abdo Z, Sandler RS, Keku TO. Molecular characterization of mucosal adherent bacteria and associations with colorectal adenomas. *Gut Microbes* 2010; **1**: 138-147 [PMID: 20740058 DOI: 10.4161/gmic.1.3.12360]
97 **Sears CL**, Pardoll DM. Perspective: alpha-bugs, their microbial partners, and the link to colon cancer. *J Infect Dis* 2011; **203**: 306-311 [PMID: 21208921 DOI: 10.1093/jinfdis/jiq061]
98 **Boleij A**, Tjalsma H. The itinerary of Streptococcus gallolyticus infection in patients with colonic malignant disease. *Lancet Infect Dis* 2013; **13**: 719-724 [PMID: 23831427 DOI: 10.1016/S1473-3099(13)70107-5]
99 **Wentling GK**, Metzger PP, Dozois EJ, Chua HK, Krishna M. Unusual bacterial infections and colorectal carcinoma--Streptococcus bovis and Clostridium septicum: report of three cases. *Dis Colon Rectum* 2006; **49**: 1223-1227 [PMID: 16845563]
100 **Medzhitov R**. Origin and physiological roles of inflammation. *Nature* 2008; **454**: 428-435 [PMID: 18650913 DOI: 10.1038/nature07201]
101 **Greten FR**, Eckmann L, Greten TF, Park JM, Li ZW, Egan LJ, Kagnoff MF, Karin M. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell* 2004; **118**: 285-296 [PMID: 15294155 DOI: 10.1016/j.cell.2004.07.013]
102 **Schwitalla S**, Fingerle AA, Cammareri P, Nebelsiek T, Göktuna SI, Ziegler PK, Canli O, Heijmans J, Huels DJ, Moreaux G, Rupec RA, Gerhard M, Schmid R, Barker N, Clevers H, Lang R, Neumann J, Kirchner T, Taketo MM, van den Brink GR, Sansom OJ, Arkan MC, Greten FR. Intestinal tumorigenesis initiated by dedifferentiation and acquisition of stem-cell-like properties. *Cell* 2013; **152**: 25-38 [PMID: 23273993 DOI: 10.1016/j.cell.2012.12.012]
103 **Femia AP**, Luceri C, Toti S, Giannini A, Dolara P, Caderni G. Gene expression profile and genomic alterations in colonic tumours induced by 1,2-dimethylhydrazine (DMH) in rats. *BMC Cancer* 2010; **10**: 194 [PMID: 20459814 DOI: 10.1186/1471-2407-10-194]
104 **Kraus S**, Arber N. Inflammation and colorectal cancer. *Curr Opin Pharmacol* 2009; **9**: 405-410 [PMID: 19589728 DOI: 10.1016/j.coph.2009.06.006]
105 **Lee SH**, Hu LL, Gonzalez-Navajas J, Seo GS, Shen C, Brick J, Herdman S, Varki N, Corr M, Lee J, Raz E. ERK activation drives intestinal tumorigenesis in Apc(min/+) mice. *Nat Med* 2010; **16**: 665-670 [PMID: 20473309 DOI: 10.1038/nm.2143]
106 **Rutter M**, Saunders B, Wilkinson K, Rumbles S, Schofield G, Kamm M, Williams C, Price A, Talbot I, Forbes A. Severity of inflammation is a risk factor for colorectal neoplasia in ulcerative colitis. *Gastroenterology* 2004; **126**: 451-459 [PMID: 14762782]
107 **Gupta RB**, Harpaz N, Itzkowitz S, Hossain S, Matula S,

Kornbluth A, Bodian C, Ullman T. Histologic inflammation is a risk factor for progression to colorectal neoplasia in ulcerative colitis: a cohort study. *Gastroenterology* 2007; **133**: 1099-105; quiz 1340-1 [PMID: 17919486]
108 **Stecher B**, Hardt WD. The role of microbiota in infectious disease. *Trends Microbiol* 2008; **16**: 107-114 [PMID: 18280160 DOI: 10.1016/j.tim.2007.12.008]
109 **Lupp C**, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, Finlay BB. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. *Cell Host Microbe* 2007; **2**: 119-129 [PMID: 18005726]
110 **Uronis JM**, Mühlbauer M, Herfarth HH, Rubinas TC, Jones GS, Jobin C. Modulation of the intestinal microbiota alters colitis-associated colorectal cancer susceptibility. *PLoS One* 2009; **4**: e6026 [PMID: 19551144 DOI: 10.1371/journal.pone.0006026]
111 **Schwabe RF**, Wang TC. Cancer. Bacteria deliver a genotoxic hit. *Science* 2012; **338**: 52-53 [PMID: 23042875 DOI: 10.1126/science.1229905]
112 **Lax AJ**. Opinion: Bacterial toxins and cancer--a case to answer? *Nat Rev Microbiol* 2005; **3**: 343-349 [PMID: 15806096]
113 **Wu S**, Rhee KJ, Albesiano E, Rabizadeh S, Wu X, Yen HR, Huso DL, Brancati FL, Wick E, McAllister F, Housseau F, Pardoll DM, Sears CL. A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. *Nat Med* 2009; **15**: 1016-1022 [PMID: 19701202 DOI: 10.1038/nm.2015]
114 **Wu S**, Morin PJ, Maouyo D, Sears CL. Bacteroides fragilis enterotoxin induces c-Myc expression and cellular proliferation. *Gastroenterology* 2003; **124**: 392-400 [PMID: 12557145]
115 **Sears CL**. Enterotoxigenic Bacteroides fragilis: a rogue among symbiotes. *Clin Microbiol Rev* 2009; **22**: 349-369 [PMID: 19366918 DOI: 10.1128/CMR.00053-08]
116 **Housseau F**, Sears CL. Enterotoxigenic Bacteroides fragilis (ETBF)-mediated colitis in Min (Apc+/-) mice: a human commensal-based murine model of colon carcinogenesis. *Cell Cycle* 2010; **9**: 3-5 [PMID: 20009569]
117 **Lara-Tejero M**, Galán JE. Cytolethal distending toxin: limited damage as a strategy to modulate cellular functions. *Trends Microbiol* 2002; **10**: 147-152 [PMID: 11864825]
118 **Bartkova J**, Rezaei N, Liontos M, Karakaidos P, Kletsas D, Issaeva N, Vassiliou LV, Kolettas E, Niforou K, Zoumpourlis VC, Takaoka M, Nakagawa H, Tort F, Fugger K, Johansson F, Sehested M, Andersen CL, Dyrskjot L, Ørntoft T, Lukas J, Kittas C, Helleday T, Halazonetis TD, Bartek J, Gorgoulis VG. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature* 2006; **444**: 633-637 [PMID: 17136093]
119 **Ge Z**, Rogers AB, Feng Y, Lee A, Xu S, Taylor NS, Fox JG. Bacterial cytolethal distending toxin promotes the development of dysplasia in a model of microbially induced hepatocarcinogenesis. *Cell Microbiol* 2007; **9**: 2070-2080 [PMID: 17441986]
120 **Buc E**, Dubois D, Sauvanet P, Raisch J, Delmas J, Darfeuille-Michaud A, Pezet D, Bonnet R. High prevalence of mucosa-associated E. coli producing cyclomodulin and genotoxin in colon cancer. *PLoS One* 2013; **8**: e56964 [PMID: 23457644 DOI: 10.1371/journal.pone.0056964]
121 **Nougayrède JP**, Homburg S, Taieb F, Boury M, Brzuszkiewicz E, Gottschalk G, Buchrieser C, Hacker J, Dobrindt U, Oswald E. Escherichia coli induces DNA double-strand breaks in eukaryotic cells. *Science* 2006; **313**: 848-851 [PMID: 16902142]
122 **Cuevas-Ramos G**, Petit CR, Marcq I, Boury M, Oswald E, Nougayrède JP. Escherichia coli induces DNA damage in vivo and triggers genomic instability in mammalian cells. *Proc Natl Acad Sci U S A* 2010; **107**: 11537-11542 [PMID: 20534522 DOI: 10.1073/pnas.1001261107]
123 **Fiorentini C**, Fabbri A, Flatau G, Donelli G, Matarrese P, Lemichez E, Falzano L, Boquet P. Escherichia coli cytotoxic necrotizing factor 1 (CNF1), a toxin that activates the Rho GTPase. *J Biol Chem* 1997; **272**: 19532-19537 [PMID: 9235957]
124 **Falzano L**, Filippini P, Travaglione S, Miraglia AG, Fabbri A, Fiorentini C. Escherichia coli cytotoxic necrotizing factor 1 blocks cell cycle G2/M transition in uroepithelial cells. *Infect Immun* 2006; **74**: 3765-3772 [PMID: 16790748]
125 **Fabbri A**, Travaglione S, Ballan G, Loizzo S, Fiorentini C. The cytotoxic necrotizing factor 1 from E. coli: a janus toxin playing with cancer regulators. *Toxins* (Basel) 2013; **5**: 1462-1474 [PMID: 23949007 DOI: 10.3390/toxins5081462]
126 **Boyer L**, Magoc L, Dejardin S, Cappillino M, Paquette N, Hinault C, Charriere GM, Ip WK, Fracchia S, Hennessy E, Erturk-Hasdemir D, Reichhart JM, Silverman N, Lacy-Hulbert A, Stuart LM. Pathogen-derived effectors trigger protective immunity via activation of the Rac2 enzyme and the IMD or Rip kinase signaling pathway. *Immunity* 2011; **35**: 536-549 [PMID: 22018470 DOI: 10.1016/j.immuni.2011.08.015]
127 **Holmes E**, Li JV, Athanasiou T, Ashrafian H, Nicholson JK. Understanding the role of gut microbiome-host metabolic signal disruption in health and disease. *Trends Microbiol* 2011; **19**: 349-359 [PMID: 21684749 DOI: 10.1016/j.tim.2011.05.006]
128 **Degirolamo C**, Modica S, Palasciano G, Moschetta A. Bile acids and colon cancer: Solving the puzzle with nuclear receptors. *Trends Mol Med* 2011; **17**: 564-572 [PMID: 21724466 DOI: 10.1016/j.molmed.2011.05.010]
129 **Carbonero F**, Benefiel AC, Gaskins HR. Contributions of the microbial hydrogen economy to colonic homeostasis. *Nat Rev Gastroenterol Hepatol* 2012; **9**: 504-518 [PMID: 22585131 DOI: 10.1038/nrgastro.2012.85]
130 **Windey K**, De Preter V, Louat T, Schuit F, Herman J, Vansant G, Verbeke K. Modulation of protein fermentation does not affect fecal water toxicity: a randomized cross-over study in healthy subjects. *PLoS One* 2012; **7**: e52387 [PMID: 23285019 DOI: 10.1371/journal.pone.0052387]
131 **Seitz HK**, Stickel F. Acetaldehyde as an underestimated risk factor for cancer development: role of genetics in ethanol metabolism. *Genes Nutr* 2010; **5**: 121-128 [PMID: 19847467 DOI: 10.1007/s12263-009-0154-1]
132 **Leonel AJ**, Alvarez-Leite JI. Butyrate: implications for intestinal function. *Curr Opin Clin Nutr Metab Care* 2012; **15**: 474-479 [PMID: 22797568 DOI: 10.1097/MCO.0b013e32835665fa]
133 **Hooper LV**, Littman DR, Macpherson AJ. Interactions between the microbiota and the immune system. *Science* 2012; **336**: 1268-1273 [PMID: 22674334 DOI: 10.1126/science.1223490]
134 **Garrett WS**, Lord GM, Punit S, Lugo-Villarino G, Mazmanian SK, Ito S, Glickman JN, Glimcher LH. Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. *Cell* 2007; **131**: 33-45 [PMID: 17923086]
135 **Mazmanian SK**, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. *Nature* 2008; **453**: 620-625 [PMID: 18509436 DOI: 10.1038/nature07008]
136 **Sansonetti PJ**, Di Santo JP. Debugging how bacteria manipulate the immune response. *Immunity* 2007; **26**: 149-161 [PMID: 17307704]
137 **Kamada N**, Seo SU, Chen GY, Núñez G. Role of the gut microbiota in immunity and inflammatory disease. *Nat Rev Immunol* 2013; **13**: 321-335 [PMID: 23618829 DOI: 10.1038/nri3430]
138 **Devkota S**, Wang Y, Musch MW, Leone V, Fehlner-Peach H, Nadimpalli A, Antonopoulos DA, Jabri B, Chang EB. Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10-/- mice. *Nature* 2012; **487**: 104-108 [PMID: 22722865 DOI: 10.1038/nature11225]
139 **Yatsunenko T**, Rey FE, Manary MJ, Trehan I, Dominguez-Bello MG, Contreras M, Magris M, Hidalgo G, Baldassano RN, Anokhin AP, Heath AC, Warner B, Reeder J, Kuczynski J, Caporaso JG, Lozupone CA, Lauber C, Clemente JC, Knights

D, Knight R, Gordon JI. Human gut microbiome viewed across age and geography. *Nature* 2012; **486**: 222-227 [PMID: 22699611 DOI: 10.1038/nature11053]

140 **Biagi E**, Candela M, Turroni S, Garagnani P, Franceschi C, Brigidi P. Ageing and gut microbes: perspectives for health maintenance and longevity. *Pharmacol Res* 2013; **69**: 11-20 [PMID: 23079287 DOI: 10.1016/j.phrs.2012.10.005]

141 **Boyle P**, Ferlay J. Mortality and survival in breast and colorectal cancer. *Nat Clin Pract Oncol* 2005; **2**: 424-425 [PMID: 16264992]

142 **Venturi V**, da Silva DP. Incoming pathogens team up with harmless 'resident' bacteria. *Trends Microbiol* 2012; **20**: 160-164 [PMID: 22390987 DOI: 10.1016/j.tim.2012.02.00]

143 **Kamada N**, Kim YG, Sham HP, Vallance BA, Puente JL, Martens EC, Núñez G. Regulated virulence controls the ability of a pathogen to compete with the gut microbiota. *Science* 2012; **336**: 1325-1329 [PMID: 22582016 DOI: 10.1126/science.1222195]

144 **Marks LR**, Davidson BA, Knight PR, Hakansson AP. Interkingdom signaling induces Streptococcus pneumoniae biofilm dispersion and transition from asymptomatic colonization to disease. *MBio* 2013; **4**: [PMID: 23882016 DOI: 10.1128/mBio.00438-13]

145 **Zhang C**, Li S, Yang L, Huang P, Li W, Wang S, Zhao G, Zhang M, Pang X, Yan Z, Liu Y, Zhao L. Structural modulation of gut microbiota in life-long calorie-restricted mice. *Nat Commun* 2013; **4**: 2163 [PMID: 23860099 DOI: 10.1038/ncomms3163]

146 **Ishikawa H**, Akedo I, Otani T, Suzuki T, Nakamura T, Takeyama I, Ishiguro S, Miyaoka E, Sobue T, Kakizoe T. Randomized trial of dietary fiber and Lactobacillus casei administration for prevention of colorectal tumors. *Int J Cancer* 2005; **116**: 762-767 [PMID: 15828052]

147 **Pool-Zobel BL**. Inulin-type fructans and reduction in colon cancer risk: review of experimental and human data. *Br J Nutr* 2005; **93** Suppl 1: S73-S90 [PMID: 15877900]

148 **Rafter J**, Bennett M, Caderni G, Clune Y, Hughes R, Karlsson PC, Klinder A, O'Riordan M, O'Sullivan GC, Pool-Zobel B, Rechkemmer G, Roller M, Rowland I, Salvadori M, Thijs H, Van Loo J, Watzl B, Collins JK. Dietary synbiotics reduce cancer risk factors in polypectomized and colon cancer patients. *Am J Clin Nutr* 2007; **85**: 488-496 [PMID: 17284748]

149 **Davis CD**, Milner JA. Gastrointestinal microflora, food components and colon cancer prevention. *J Nutr Biochem* 2009; **20**: 743-752 [PMID: 19716282 DOI: 10.1016/j.jnutbio.2009.06.001]

150 **Ventura M**, Turroni F, Motherway MO, MacSharry J, van Sinderen D. Host-microbe interactions that facilitate gut colonization by commensal bifidobacteria. *Trends Microbiol* 2012; **20**: 467-476 [PMID: 22902802 DOI: 10.1016/j.tim.2012.07.002]

151 **Candela M**, Perna F, Carnevali P, Vitali B, Ciati R, Gionchetti P, Rizzello F, Campieri M, Brigidi P. Interaction of probiotic Lactobacillus and Bifidobacterium strains with human intestinal epithelial cells: adhesion properties, competition against enteropathogens and modulation of IL-8 production. *Int J Food Microbiol* 2008; **125**: 286-292 [PMID: 18524406 DOI: 10.1016/j.ijfoodmicro.2008.04.012]

152 **Ruas-Madiedo P**, Medrano M, Salazar N, De Los Reyes-Gavilán CG, Pérez PF, Abraham AG. Exopolysaccharides produced by Lactobacillus and Bifidobacterium strains abrogate in vitro the cytotoxic effect of bacterial toxins on eukaryotic cells. *J Appl Microbiol* 2010; **109**: 2079-2086 [PMID: 20846331 DOI: 10.1111/j.1365-2672.2010.04839.x]

153 **Carey CM**, Kostrzynska M, Ojha S, Thompson S. The effect of probiotics and organic acids on Shiga-toxin 2 gene expression in enterohemorrhagic Escherichia coli O157: H7. *J Microbiol Methods* 2008; **73**: 125-132 [PMID: 18328583 DOI: 10.1016/j.mimet.2008.01.014]

154 **Bayoumi MA**, Griffiths MW. In vitro inhibition of expression of virulence genes responsible for colonization and systemic spread of enteric pathogens using Bifidobacterium bifidum secreted molecules. *Int J Food Microbiol* 2012; **156**: 255-263 [PMID: 22541391 DOI: 10.1016/j.ijfoodmicro.2012.03.034]

P- Reviewers: Abdel-Salam OME, Beyaert R, Cairo G, Teramoto-Matsubara OT **S- Editor**: Gou SX **L- Editor**: A **E- Editor**: Wu HL

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.923

World J Gastroenterol 2014 January 28; 20(4): 923-942
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Cancer stem cells in colorectal cancer from pathogenesis to therapy: Controversies and perspectives

Caterina Fanali, Donatella Lucchetti, Marisa Farina, Maddalena Corbi, Valerio Cufino, Achille Cittadini, Alessandro Sgambato

Caterina Fanali, Donatella Lucchetti, Marisa Farina, Maddalena Corbi, Valerio Cufino, Achille Cittadini, Alessandro Sgambato, Istituto di Patologia Generale, Università Cattolica del Sacro Cuore, 00168 Rome, Italy

Author contributions: Fanali C, Lucchetti D and Farina M contributed equally to this work; all authors contributed to manuscript preparation; Sgambato A supervised the work and was responsible for the final editing.

Correspondence to: Alessandro Sgambato, MD, PhD, Istituto di Patologia Generale, Università Cattolica del Sacro Cuore, Largo Francesco Vito 1, 00168 Rome, Italy. asgambato@rm.unicatt.it

Telephone: +39-6-3016619 Fax: +39-6-3012753

Received: October 15, 2013 Revised: November 20, 2013
Accepted: January 2, 2014
Published online: January 28, 2014

Abstract

Colorectal cancer remains one of the most common and lethal malignancies worldwide despite the use of various therapeutic strategies. A better understanding of the mechanisms responsible for tumor initiation and progression is essential for the development of novel, more powerful therapies. The traditional, so-called "stochastic model" of tumor development, which assumes that each cancer cell is tumorigenic, has been deeply challenged during the past decade by the identification of cancer stem cells (CSCs), a biologically distinct subset of cells within the bulk of tumor mass. This discovery led to the development of the hierarchical model of tumorigenesis which assumes that only CSCs have the ability to initiate tumor growth, both at primary and metastatic sites. This model implies that the elimination of all CSCs is fundamental to eradicate tumors and that failure to do so might be responsible for the occurrence of relapses and/or metastases frequently observed in the clinical management of colorectal cancer patients. Identification and isolation of CSCs is essential for a better understanding of their role in the tumorigenetic process and for the development of CSC-specific therapies. Several methods have been used for this purpose and many efforts have been focused on the identification of specific CSC-surface markers. This review provides an overview of the proposed roles of CSC in human colorectal tumorigenesis focusing on the most important molecules identified as CSC-specific markers in colorectal cancer and on the potential strategies for the development of CSC-targeted therapy.

Key words: Colorectal cancer; Cancer stem cells; Tumorigenesis; Cancer therapy; Prognostic marker

Core tip: A better understanding of the mechanisms responsible for tumor initiation and progression is essential for the development of novel, more powerful therapies for colorectal cancer patients. In this paper, we review the basic concepts of both the traditional "stochastic", and of the more recent, "hierarchical" models of tumor development. We then introduce the so-called cancer stem cells (CSCs) and provides an overview of the proposed roles of CSCs in human colorectal tumorigenesis focusing on the most important molecules identified as CSC-specific marker in colorectal cancer and on the potential strategies for the development of CSC-targeted therapy.

Fanali C, Lucchetti D, Farina M, Corbi M, Cufino V, Cittadini A, Sgambato A. Cancer stem cells in colorectal cancer from pathogenesis to therapy: Controversies and perspectives. *World J Gastroenterol* 2014; 20(4): 923-942 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/923.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.923

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies in Western countries and, although it can cause symptoms at a very early stage and can be easily detected and treated by resection, it remains the second leading cause of cancer-related death in Europe and the third in the United States with a median survival time ranging from less than one to more than five years depending on the stage of disease at the diagnosis and the surgical techniques and/or chemotherapy used, especially for metastatic CRC[1]. Several studies have underlined the role of environmental and lifestyle factors in colorectal carcinogenesis showing an increase in CRC incidence in parallel with economic development and adoption of a western lifestyle in several countries.

CRC originates from epithelial cells lining the gastrointestinal tract which undergo sequential mutations in specific DNA sequences that disrupt normal mechanisms of proliferation and self-renewal[2]. The intestinal tract consists of the small intestine (duodenum, jejunum and ileum) and the large intestine or colon, which comprises the cecum, ascending, transverse and descending colon, sigmoid colon, rectum and anal canal. The innermost layer of the colon wall (mucosa) is lined by an absorptive and secretory columnar epithelium which is folded into finger-like invaginations incorporated in the submucosa connective tissue to form the functional unit of the intestine, the crypts of Lieberkühn (Figure 1). Normal human colon consists of millions of crypts containing about 2000 cells and comprising the differentiated cell lineages (enterocytes, enteroendocrine cells and goblet cells). A fourth differentiated type, the Paneth-like cells, resides at the bottom of colon crypts and has been shown to synthesize and secrete a variety of antimicrobial factors[3]. Differentiated colon epithelial cells are subjected to a massive turnover throughout life, being replaced approximately every 5 d. The ability to maintain tissue homeostasis is provided by a subset of self-renewing undifferentiated, multipotent stem cells which generate transit-amplifying cells, committed progenitors[3]. These cells lie towards the bottom of the crypt in the proliferative zone and through an asymmetric division are responsible for generating all epithelial cell types along the crypt-villus axis. The number of long-lived stem cells per each crypt is commonly estimated to be between 4 and 6 cells even if the precise number and what controls their numbers remain uncertain (Figure 1). Two distinct populations of putative stem cells have been identified at the base of intestinal crypts. A population is marked by the expression of the G-protein receptor Lgr5, a Wnt gene target, and positioned just above the Paneth cells at the crypt base, while the other resides at +4 position from the bottom of the crypt and are marked by the expression of the polycomb group gene Bmi1 and the telomerase reverse transcriptase, Tert[4,5]. Both cell types have been demonstrated to fulfill the criteria for stem cells (pluripotency and self-renewal capacity)[4,5]. Several studies are trying to understand whether their stem cell characteristics are

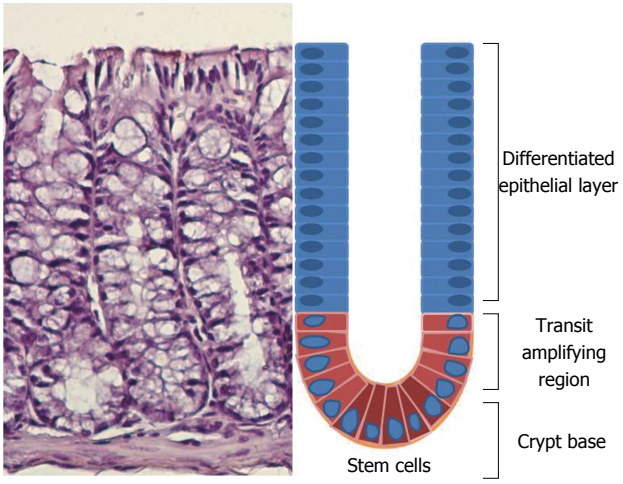


Figure 1 Schematic representation of an individual colon crypt showing the position of different cell types. Stem cells lie at the bottom of the crypt and through an asymmetric division are responsible for generating all epithelial cell types along the crypt-villus axis.

intrinsically determined or determined by the environmental niche. It is widely accepted, however, that stem cell niches are formed by cellular components and extracellular matrix which create a special microenvironment important for the maintenance of stem cells properties, protect stem cells from differentiating and apoptotic stimuli and regulate the balance between proliferation and differentiation through direct interaction and secretion of various cytokines and growth factors[6]. The stem cells self-renewal and differentiation are also influenced by components in the crypt lumen derived from bacteria or epithelial cells as well as by morphogenetic factors secreted by intestinal sub-epithelial myofibroblasts[7].

Mounting evidence suggests that stem cells might play an important role in the process of tumor development being able to acquire a tumorigenic potential and giving rise to the so-called cancer stem cells whose potential role as tumor initiating cells as well as targets of cancer therapies is discussed in this review.

Models of colorectal tumorigenesis

CRC has been an ideal model to study the malignant progression because different phases of the same malignancy often coexist within the same patient and have provided basic information concerning human tumorigenesis. Although most of the CRCs are sporadic, a small percentage arises in the setting of inherited syndromes, such as familial adenomatous polyposis (FAP), juvenile polyposis syndrome (JPS) and hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome), which have been extremely useful for our understanding of human colorectal tumorigenesis. The study of these hereditary cancer syndromes, as well as of sporadic CRC, has led to a detailed knowledge of the sequence of genetic mutations underlying CRC development with the formulation of a model of multistep carcinogenesis which has been subsequently extended to the majority of human can-

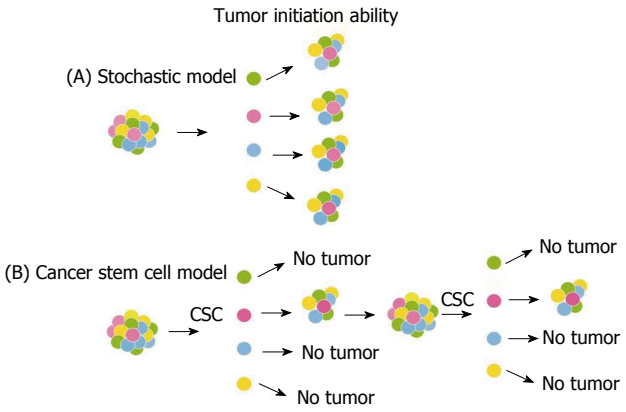


Figure 2 Models of tumor development. (A) Stochastic model: every cancer cell isolated from the bulk tumor is tumorigenic and thus has the ability to proliferate extensively and initiate tumor growth. (B) Cancer stem cell (CSC) model: only a rare subpopulation of undifferentiated cells has the unique biological properties necessary for tumor initiation, maintenance, and spreading.

cers[8]. It is now widely accepted that, regardless of the starting event, CRC is the end result of a variable concatenation of genetic alterations that lead a normal colonic epithelial cell to transform into a colon cancer cell. According to the model of colorectal tumorigenesis, initially proposed by Fearon *et al*[8] (also known as the adenoma-cancer sequence), CRC development occurs through a series of steps morphologically identifiable: initially there is localized proliferation of the colon epithelium with the formation of small adenomas which progressively grow with dysplasia and ultimately progress into invasive carcinomas. Most of the CRC is characterized by a dysfunctional regulation of the Wnt/β-catenin pathway, essential for the development of the normal colonic mucosa[9]. About 80% of patients with FAP have loss or mutation in the APC (adenomatous polyposis coli) gene which encodes a protein that participates to the formation of a complex that regulates the stability of β-catenin. In the absence of Wnt ligand this complex retains the β-catenin which is phosphorylated and degraded by the proteasome. When the APC molecule is mutated, the cytosolic β-catenin levels are stabilized and the protein can then accumulate in the nucleus where it serves as a coactivator for the Tcf family of transcription factors which activate the expression of specific target genes including some metalloproteinases, the fibronectin and oncogenes such as c-myc and cyclin D1[10]. The study of JPS, a condition that predisposes to hamartomatous gastrointestinal polyps formation, has revealed the important role of SMAD/BMP (bone morphogenetic protein) in intestinal architecture. JPS is due to germline mutations in the SMAD4 gene in 15%-20% of cases and to mutations in the gene encoding BMP receptor 1A in 25%-40% of cases. SMAD4 is an intracellular signal transducing transcription factor shared by the Transforming growth factor β, activin and BMP pathways. BMP family ligands are expressed by the villus mesenchyme, while epithelial cells display nuclear phosphorylated SMADs, implicating these cells as terminal recipients of the signal[11]. Wnt and BMP pathways interact to control intestinal stem cells self-renewal through the PTEN-Akt pathway that helps to control the nuclear localization of the Wnt pathway effector β-catenin[12]. Interaction between Wnt and Notch pathway, which maintains proliferative cells in normal scripts, is also deregulated in tumorigenesis[9]. Wnt signaling, Hedgehog, BMP, Notch and Platelet-derived growth factors are involved in the process of epithelial to mesenchymal transition and invasion[7].

According to the traditional model of carcinogenesis a tumor may arise from any cell of the body following a series of mutations conferring them an unlimited proliferation potential. The resulting mutated progeny is subject to additional mutations, due to genetic instability, and epigenetic changes, promoting the appearance of a genetically heterogeneous tumor mass.

This view has been initially integrated in the so-called "stochastic model" of tumor development which assumes that each cancer cell isolated from the bulk tumor is tumorigenic and thus has the ability to proliferate extensively and regenerate a tumor with the same characteristics of the original tumor when injected in immunodeficient mice (Figure 2).

The presence of cell types with various degrees of differentiation within human CRC[13] and of a stem cells overpopulation at the botton crypt during the process of adenoma development in patients with FAP, has suggested a hierarchical model of CRC development as opposed to the stochastic model. According to this model, only a small fraction of tumor cells would be able to support the neoplastic proliferation, the part that retains the characteristics of stem cells and that in itself has a unlimited proliferative potential. The tumors would be organized as a normal tissue with a rare subpopulation of undifferentiated cells having the unique biological properties necessary for tumor initiation, maintenance, and spreading[14] (Figure 2). These cancer cells displaying stemness features have been defined cancer stem cells (CSCs) and, similarly to normal stem cells, would be located in a niche with mesenchymal cells that would ensure their survival in a secure environment, regulating their proliferation through secretion of soluble factors[15]. According to this model, these slow proliferating CSCs display self-renewal, unlimited proliferative potential and multipotency and would be responsible for tumor initiation and development as well as local relapses and metastases. Moreover, CSCs would be highly resistant to traditional antineoplastic agents due to the expression of detoxifying enzymes, drug transporters and DNA repair mechanisms[15].

The origin of CSCs remains unclear and it is still object of a debate whether they derive from more mature cells that reacquire stem cell properties during tumor formation or are the direct progeny of mutated stem cells[15] (Figure 2). The discovery of stem cells in the majority of normal tissues, including colon crypts, supports the hypothesis that normal stem cells might represent a possible target for tumorigenic mutations and the origin of CSCs due to both their longevity and their ability

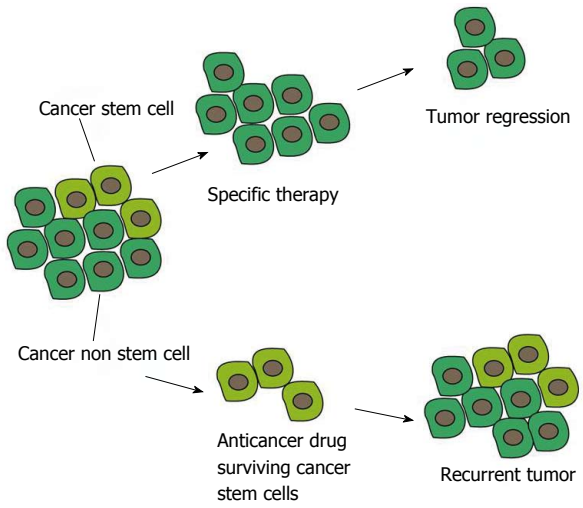


Figure 3 Advantages of a cancer stem cell-specific therapy compared to conventional anticancer therapies. The current anticancer drugs wipe out most of the bulk population but the surviving cancer stem cells (CSCs) can repopulate the tumor. Specific targeting of CSCs is essential for regression and complete eradication of the tumor.

to self-renewal. Furthermore, the fact that despite the emergence of new targeted agents and the use of various therapeutic combinations, none of the options currently available is curative for patients with CRC strengthens the model of CSCs and supports the hypothesis that most of the currently available therapies only target the bulk of the tumor mass (mainly constituted by proliferating cells) while sparing the rare, quiescent CSCs which would be able to re-initiate tumor growth thus giving origin to both recurrences and metastases (Figure 3). This hypothesis supports the need of a better characterization of CSCs with the aim to develop CSCs-specific therapies which might represent a great advantage in the fight against cancer.

COLORECTAL CANCER STEM CELLS MARKERS

Several methods have been proposed in the last years for the identification and isolation of CSCs but some of them require a great technical expertise, are extremely time-consuming or involve the use of animals. Thus, many efforts have been focused on the identification of specific CSCs-surface markers which would allow the identification, and likely the isolation, of CSCs using easier antibody-based techniques such as immunostaining, *Fluorescence-activated cell sorting* (FACS) analysis, cell sorting, immunomagnetic separation, *etc*. One of the first CSCs markers identified in human CRC was the CD133, a pentaspan transmembrane glycoprotein which was shown to specifically mark tumor-initiating cells within the bulk of human CRC[16,17]. In the same period Dalerba *et al*[18] found that $CD133^+$ cells population also express other specific stem cell antigens such as EpCAM, CD44 and CD166 which can help to identify CSCs while a subsequent study showed that CD133 colon cancer cells spheroids grown *in vitro* also expressed Msi-1[18]. Other potential markers of CRC stem cells have been more recently identified including CD29, CD24 and Lgr5[19-21] (Table 1).

CD133/Prominin-1

Human CD133, also known as Prominin-1, is a 120 kDa cholesterol-interacting pentaspan-transmembrane glycoprotein that belongs to the Prominin family. CD133 protein consists of an extracellular N-terminal domain, a cytoplasmic C-terminus that contains five tyrosine residues including a tyrosine phosphorylation consensus site, two small cysteine-rich cytoplasmic loops and two large extracellular loops containing four consensus sequences for N-linked glycosylation[22] (Figure 4).

CD133 was first recognized as a surface protein marker of a subset of hematopoietic stem cells and progenitor cells[22] and of bone marrow-derived circulating endothelial progenitors involved in postnatal angiogenesis, inflammation and tissue regeneration[23,24]. Subsequently, it was identified in several human normal tissues and on CSCs from a variety of solid tumors including brain, colon, liver, lung and prostate neoplasms[23,25].

Two studies first identified CD133 as a marker for stem cells in CRC. Ricci-Vitiani *et al*[16] showed the tumorigenic potential of $CD133^+$ human CRC cells and evidenced their ability to engraft and give rise to visible tumors in immunodeficient mice even after serial transplantations. Simultaneously, O' Brien *et al*[17] demonstrated an enrichment of more than 200-fold of cancer-initiating cells in the subsets of $CD133^+$ cells isolated from human CRC samples compared to unsorted cancer cell populations. Moreover, they showed that liver metastases are enriched with a population of $CD133^+$ cancer cells, a finding also confirmed by our group[26], and observed that tumor xenografts generated from $CD133^+$ cells reproduced the histological features of the original tumor[17].

CD133 is concentrated in plasma membrane protrusions, containing lipid rafts, and more recently several studies have suggested a link between the release of CD133 contained in the membrane vesicles and cellular differentiation, proving that CD133 might play a key role in maintaining stem cell properties[27,28]. However, the discussion on the effective value of CD133 and its usefulness as a CSC biomarker is still controversial because other studies have shown that the $CD133^-$ population of CRC cells is also able to initiate tumor growth in immunodeficient mice[29]. More recently, Feng *et al*[30] proposed another possibility to explain the central issue of the debate, showing that the sorted $CD133^+$ and $CD133^-$ SW620 colon cancer cells can undergo a conversion between the two cell subsets, this resulting in contradictory data. Moreover, Hsu *et al*[31], showed that the exposure to environmental stress, hypoxia and cell-adhesion free condition, promoted switching of $SW620CD133^-$ cells to $SW620CD133^+$ cells while exposure to ECM components promoted switching of $SW620CD133^+$ to $SW620CD133^-$ cells. The switching between the two

Table 1 Cell surface and intracellular molecules suggested as putative cancer stem cell markers in colorectal cancer and their most important features

Marker	Other name	Gene location[1]	Function	Ref.
CD133	Prominin-1, AC133	Chr 4 (p15.32)	Encoding of a pentaspan transmembrane glycoprotein which binds cholesterol in cholesterol-containing plasma membrane microdomains	[22]
CD44	PGP-1, HUTCH-1, GP90, EPICAN, CDW44, MIC4	Chr 11 (p13)	Cell adhesion molecule; involved in lymph node homing and lymphocyte activation	[55,56]
EpCAM	ESA, CD326, MK- 1, KSA, HEA125, BerEp4, 17-1A, GA733-2, KS1/4, EGP-2, EGP34, TROP-1	Chr 2 (p21)	Epithelial cell adhesion molecule	[70]
CD24	HSA	Chr 6 (q21)	Mucin-like cell adhesion molecule	[76]
CD29	B1 Integrin	Chr 10 (p11.2)	Receptor for extracellular matrix proteins; involved in regulation of cell migration, proliferation, survival, differentiation and death	[82]
Lgr5	GPR49	Chr 12 (q22-q23)	Receptor for R-spondin proteins; marker for adult stem cells	[89,90]
CD166	ALCAM	Chr 3 (q13.1)	Cell adhesion molecule	[96]

[1]From http://www.ncbi.nlm.nih.gov/gene.

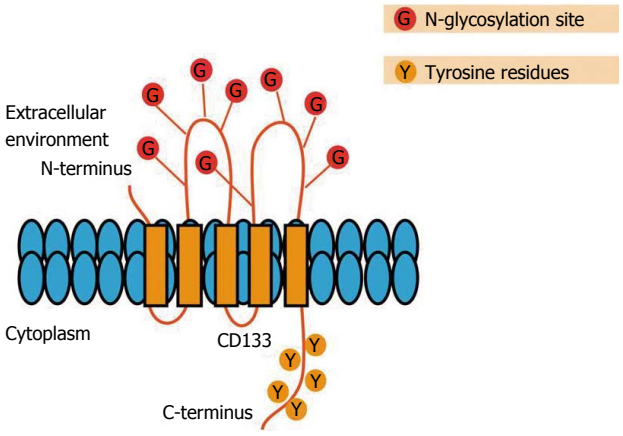


Figure 4 Schematic representation of the CD133 molecule. CD133 consists of an extracellular N-terminal domain, a cytoplasmic C-terminus containing five tyrosine residues, two small cysteine-rich cytoplasmic loops and two large extracellular loops, each containing four consensus sequences for N-linked glycosylation.

Table 2 Prognostic value of CD133

Marker	Prognostic value	Ref.
CD133	Worse outcome and higher risk of metastasis	[32]
	Independent prognostic marker for overall survival	[33]
	Association with CSC resistance to 5FU-based chemotherapy in CRC	[34]
	Association with resistance to conventional radiotherapy in CRC	[35]
	Prediction of distant recurrences after chemoradiotherapy in colon cancer patients	[35]
	High tumorigenicity of $CD133^+$ CRC cells compared to $CD133^-$ cells due to their interaction with CAFs by paracrine signaling axis of CXCR4-SDF1	[36]
	Risk factor for poor overall survival in stage Ⅱ and Ⅲ in colon cancer patients	[37]
	Relationship with K-Ras and B-Raf mutations in CRC patients	[38]

CSC: Cancer stem cell; CRC: Colorectal cancer.

subpopulations might be important for the adaptation to the microenvironment in tumor colonization (Figure 5). On this base, the current concept that CSCs unidirectionally differentiate into non stem cells could be challenged by the findings that non CSCs can convert in a stem-like state within the tumor depending on environmental stimuli[31].

Although the exact functional role of CD133 is still controversial, several studies have addressed its potential diagnostic and prognostic value as well as its intracellular signaling pathways. Several papers investigated the prognostic role of CD133 expression by immunohistochemistry and showed a high prognostic relevance for colon cancer progression and metastasis. Kojima *et al*[32] linked CD133 overexpression with a worse outcome and a higher risk of metastasis in CRC patients, a finding confirmed by Horst and others who showed that CD133 expression is an independent prognostic marker for overall survival[32,33]. Ong *et al*[34] demonstrated that high expression of CD133 is associated with resistance of CSC to 5-FU-based chemotherapy as well as with a significant worse survival. Moreover, $CD133^+$ cells have been shown to be more resistant to conventional radiation therapy, thus suggesting that post-chemoradiotherapy CD133 expression may predict the risk of distant recurrence and poor survival in radiotherapy-treated CRC patients[35].

Chao *et al*[36] proposed that $CD133^+$ CRC cells are more tumorigenic than $CD133^-$ cells due to their interaction with carcinoma-associated fibroblasts in tumor microenvironment by the paracrine signaling axis CXCR4-SDF-1 (Figure 6). This evidence was confirmed by Zhang *et al*[37] who showed that the co-expression of CXCR4 and CD133 on tumor cells was an independent risk factor for poor overall survival in stage Ⅱ and Ⅲ CRC patients. The prognostic role of CD133 in CRC patients was confirmed by Kemper *et al*[38] who showed a relationship between CD133 expression and the presence of mutations in K-Ras or B-Raf genes and suggested that CD133 might be regulated by the Ras-Raf-Mek-Erk pathway (Table 2).

The study of Mohammadi *et al*[39] was the first to evaluate the expression of CD133 in premalignant

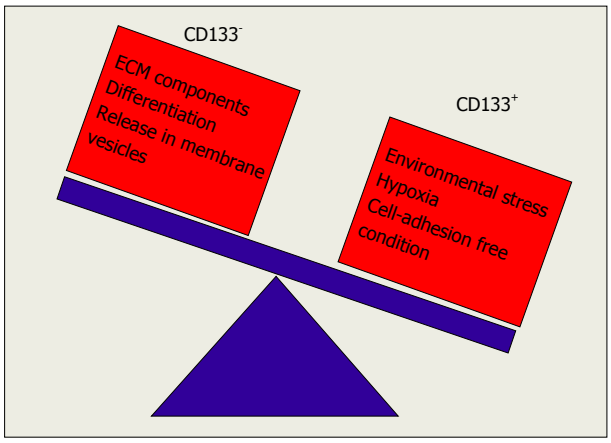


Figure 5 Signals regulating CD133 expression levels. The exposure to environmental stress, hypoxia and cell-adhesion-free condition promotes switching of CD133$^-$ to CD133$^+$ cells while exposure to ECM components promotes switching of CD133$^+$ to CD133$^-$ cells.

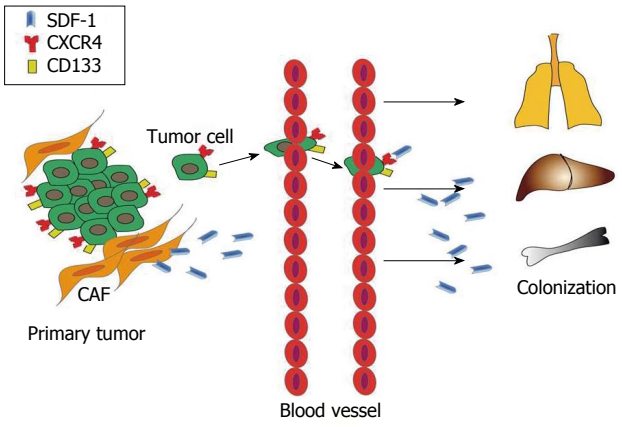


Figure 6 Possible role of the interaction between CXCR4/CD133 cancer cells and SDF-1 ligands. The SDF-1 ligand secreted by carcinoma-associated fibroblasts (CAF) in tumor microenvironment interacts with CXCR4/CD133 expressing cancer cells and could drive primary tumor cells towards metastatic sites.

Table 3 Prognostic value of CD44

Marker	Prognostic value	Ref.
CD44	Association of CD44 downregulation with a lower metastatic potential of CRC cells	[61]
	Association of CD44 downregulation with a higher metastatic and migratory potential of CRC cells	[62]
	Association with a reduced survival	[63]
	Correlation of CD44 loss with a higher tumor aggressiveness	[64]
	Relation with CRC cells proliferation but not with patients outcome	[65]
	Association with lymph node involvement and invasion depth	[66]
	Unfavorable prognostic factor for overall survival in advanced CRC	[66]
	Correlation of CD44 loss with advanced tumor stage, vascular invasion, lymph node involvement and infiltrating tumor border	[67]
	Association of CD44 loss in the lesion invasive front with adverse outcome of CRC patients	[67]

CRC: Colorectal cancer.

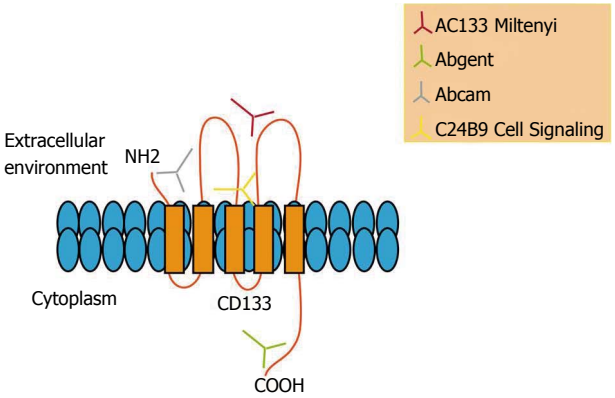


Figure 7 Epitopes recognized by different antibodies on CD133 molecule.

colorectal lesions such as non-dysplastic serrated polyps that comprise hyperplastic polyps (HP) and the non-dysplastic subset of sessile serrated adenoma-polyp-lesions (SSA/P/L) and its borderline variant. They showed that SSA/P/L and its borderline variant significantly express higher levels of CD133 than HP. They demonstrated that this premalignant colorectal lesion could be easily identified by determining the CD133 immunoprofile thus suggesting the usefulness of CD133 immunohistochemical evaluation in the diagnostic clinic routine[39]. Our group also reported that CD133 expression in human CRC is an independent risk factor associated with patient survival in multivariate analyses[40]. However, overall the data available in the literature do not allow a definitive and clear-cut assessment of the potential prognostic significance of CD133 expression which, as previously mentioned, is also the result of different antibodies, protocols and scoring criteria used for the evaluation of CD133 expression levels in clinical samples[41]. Therefore, some controversies could be a consequence of using different types of primary anti-CD133 antibodies to identify CD133$^+$ cells: most of the studies use the anti-human CD133/clone AC133 monoclonal antibody (Miltenyi) recognizing a glycosylated extracellular epitope of the CD133 molecule which can be downregulated independently from the corresponding mRNA and protein[28]. However, several other antibodies are available and are indistinctly used although they recognize different epitopes of the molecule and could give different results[41] (Figure 7).

The role of CD133 in colorectal tumorigenesis has been also investigate in mice. Zhu *et al*[42], demonstrated that in a murine model of colorectal tumorigenesis the endogenous activation of the Wnt signaling was associated with a marked expansion of CD133$^+$ cells which replaced normal mucosa architecture giving rise to neoplastic lesions. Our group analyzed by immunohistochemistry the expression of CD133 in a mouse model of colitis-related colon tumorigenesis induced by a combined treatment with azoxymethane and dextran sodium sulphate. In normal tissues rare scattered positive cells were detectable at the bottom of the crypts. The percentage of

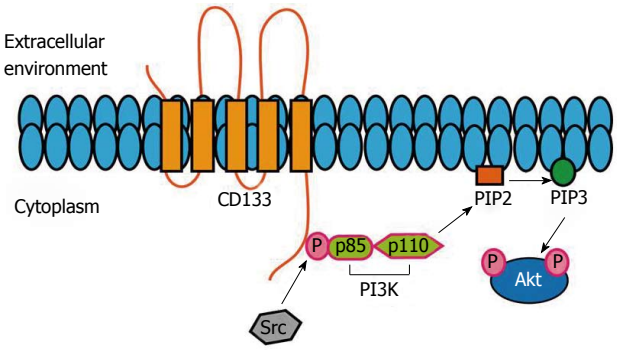


Figure 8 Potential molecular pathways associated with CD133. The phosphorylation of the tyrosine 828 is involved in the binding to p85 (PI3K regulatory subunit) and in the subsequent activation of PI3K/Akt pathway, which, finally, promotes the self-renewal and tumor formation of CSCs. CSCs: Cancer stem cells.

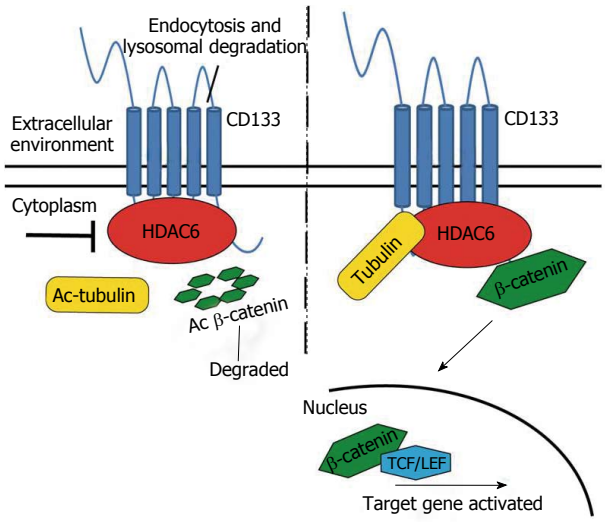


Figure 9 Schematic representation of HDAC6-mediated regulation of CD133 expression. HDAC6 physically binds to CD133 and stabilizes the β-catenin that in the nucleus promotes the activation of its target genes. Ac: Acetylated.

positive cells significantly increased in dysplastic lesions and appeared to progressively decrease in the passage from dysplasia to adenoma and then to cancer although remaining constantly higher than in adjacent normal tissues[43]. Overall these data, considered together with Mohammadi findings, suggest that upregulation of CD133 expression likely occurs at early stages and contributes to the entire process of colon tumorigenesis[43,44].

The identification of the potential molecular pathways involved in the enhanced tumorigenicity associated with CD133 expression is of great interest since it could be useful to identify and develop a targeted anticancer therapy against the CSC population. It has been reported that the CD133 glycoprotein is phosphorylated on the tyrosine-828 and tyrosine-852 residues within its C-terminal cytoplasmic tail, in a Src kinase-dependent manner. The tyrosine-828, upon phosphorylation could serve as a binding site for the SH2 domains of tyrosine kinases[44]. The phosphorylation of tyrosine-852 does not require the binding to the SH2 domains. In this regard, Wei *et al*[45] showed that, in the glioma CSCs, the phosphorylation of the tyrosine 828 is involved in the binding to p85 (PI3K regulatory subunit) and in the subsequent activation of PI3K/Akt pathway, which, finally, promotes the self-renewal and tumor formation of CSCs (Figure 8). Wang *et al*[46] reported that the inactivation of Akt and Erk pathways prevented the preferential survival of $CD133^+$ colon cancer cells isolated from primary CRC and decreased their tumorigenicity. Moreover, the down-regulation of Akt and Erk by short interfering RNAs attenuated the colony formation ability of $CD133^+$ cells[46]. More recently, it has been also showed that Silibinin, a chemo-preventive agent proved to be effective in several types of cancer, acts by inhibiting the PP2Ac/Akt/mTOR pathway which is associated with a reduction of CD133 expression in CRC spheroid cultures[47]. The Wnt signaling cascade plays various roles in stem cell maintenance, cell proliferation, differentiation and apoptosis and the deregulation of the Wnt pathway is associated with cancers. Corbo *et al*[48] reported a positive correlation among CD133 expression, Wnt pathway activation and increased SRp20 expression (splicing factor, a newly identified target gene of the Wnt/β-catenin pathway) in colon cancer cells. Furthermore, fibronectin, a major extracellular matrix glycoprotein, has been shown to be required for maintaining CD133 and CD44 positive subpopulations and tumorigenic capacity of CRC cells by activation of Wnt/β-catenin and its downstream integrin-Fak-Erk signaling pathways[49].

The regulation of CD133 expression is not fully understood but several evidences suggest the existence of multiple mechanisms involved in the regulation of CD133 expression and/or activity. As previously mentioned, CD133 is concentrated in plasma membrane protrusions and the release of CD133-containing membranous vesicles has been shown to contribute to the regulation of CD133 expression levels in several cell types[27]. Post translational modifications (*i.e.*, glycosylation) have been also suggested to play a role in the regulation of CD133 activity and its significance as a CSCs marker[28]. Indeed, it has been proposed that AC133, one of the most important epitopes of the molecule, rather than the entire molecule itself, might be important as CSCs marker.

Mak *et al*[50] proposed that CD133 expression is also regulated at a protein level by the deacetylase HDAC6, whose interaction with CD133 prevents its degradation by deacetylating α-tubulin and promotes the deacetylation of β-catenin and the activation of its signaling pathway. Moreover, they demonstrated that the inhibition of HDAC6 promotes CD133 trafficking into endosomes by increasing α-tubulin acetylation and is associated with β-catenin degradation (Figure 9).

The regulation of CD133 gene expression is also still poorly understood. Hypoxia and increased expression of hypoxia-inducible factors (HIFs) are associated with tumor progression and patient mortality in many solid tumors such as colorectal cancer, in which high expression levels of HIF-1α have been associated with poor

prognosis[51]. Ohnishi *et al*[52] suggested that the activity of one of the putative CD133 promoters (P5) is regulated by HIFs in human embryonic kidney and colon cancer cells. In particular, the CD133 promoter P5 appears to be activated by HIF-1α and HIF-2α through one of two E-twenty six (ETS) binding sites. This finding is consistent with the observation of Mao *et al*[53] that the $CD133^+$ populations in human CRC specimens express more HIF-1α than the $CD133^-$ cell population. Moreover, they engrafted human CRC specimens in BALB/c nu/nu mice and demonstrated that the majority of the $CD133^+$ population in tumor xenografts was localized in the hypoxic region. The same Authors also demonstrated that the percentage of $CD133^+$ cells increased following chemotherapy (5-fluorouracil, oxaliplatin or 5-fluorouracil plus oxaliplatin) thus indicating that $CD133^+$ cells were less sensitive to drugs than the $CD133^-$ counterparts and that the tumor hypoxic region could be associated with chemotherapeutic resistance of colon CSCs[53]. The possibility that potential epigenetic mechanisms might be also involved in the regulation of CD133 expression in CRC has been suggested by Yi *et al*[54] who described an abnormal DNA hypermethylation in a CpG island in the promoter region of the CD133 gene in colon cancer cells but further studies are required to definitively address this type of regulation for CD133 expression.

All these findings suggest a potential key role of CD133 in the initiation and progression of human CRC and support its value as a possible prognostic and diagnostic marker in CRC. The knowledge of the regulatory mechanisms upstream of CD133 and of the molecular mechanisms activated downstream could be useful in the development of targeted drugs specifically directed against CSCs, in an attempt to prevent recurrence, metastasis and chemotherapy resistance in CRC patients.

CD44

CD44 is member of a family of transmembrane proteins that include at least 20 variants resulting from a single gene by both alternative splicing and post-translation modifications[55]. The human CD44 gene includes 20 exons: exons 1-5 and exons 16-20 form a mRNA that code for a standard form of CD44 which is present in all tissues (CD44s); exons 6-15 are subject to alternative splicing that, in theory, may give life to more than 1000 variant isoforms of CD44 (CD44v)[56]. The standard isoform of human CD44 protein contains 363 amino acids and is formed by three regions: the extracellular (270 aa), the transmembrane (21 aa) and the C-terminal cytoplasmic (72 aa) domains. The presence of variable exons, mainly involving the extracellular domain, confers to CD44 a large variability of biological functions, that contributes to tumorigenicity when CD44 is expressed on tumor cells[56].

CD44 is a cell adhesion molecule that allows cell-cell and cell-ECM interactions through the binding to its principal ligand, hyaluronic acid (HA). It is also involved in lymph node homing and lymphocyte activation, myelopoiesis, lymphopoiesis, and angiogenesis[56]. CD44s, the smallest CD44 isoform that lacks variant exons, is abundantly expressed by both normal and cancers cells, whereas the CD44v isoforms that contain a variable number of exon insertions are mainly expressed by cancer cells[56].

CD44 is submitted to sequential proteolytic cleavages in the ectodomain and intramembranous domain, key events for the CD44 dependent cell-matrix interaction and signaling pathway. Cleavage of CD44 ectodomain is regulated by multiple stimuli such as extracellular Ca^{2+} influx, activation of protein kinase C or Ras and is mediated by membrane-associated matrix metalloproteinases. The release of the soluble ectodomain (soluble CD44) regulates cell attachment and migration and induces the intramembranous domain cleavage, mediated by the presenilin (PS)-dependent γ-secretase, that releases the intracellular domain of CD44 (CD44-ICD). CD44-ICD translocates to the nucleus, where it activates gene transcription, including CD44 itself, via binding to TPA-responsive elements[57] (Figure 10).

CD44 has been proposed as CSCs marker of several solid tumors, including breast, pancreas, head and neck, non-small cell lung, hepatocellular and colon cancers[18,56]. $CD44^+$ CRC cells display a greater ability to form colonies *in vitro* and a higher tumorigenicity *in vivo* compared to $CD44^-$ cells. Moreover, only $CD44^+$, but not $CD44^-$ CRC cells are able to retain the morphological and phenotypic characteristics of tumor lesions from which they were derived following serial transplantations[58]. The association of CD44 with CD54 (a member of the immunoglobulin super-family also called intercellular adhesion molecule-1) has been shown to specifically identify rectal CSC displaying the ability to self-renew *in vivo* and *in vitro*, form spheres and recapitulate tumor bulk[59].

CD44 expression is regulated by the Wnt signaling pathway *via* β-catenin. In fact, activation of β-catenin/Tcf-4 signaling in intestinal tumors is associated with CD44 overexpression and deletion of CD44 in APC Min/+mice inhibits the initiation of tumors[60]. CD44 appears to be essential for stemness maintenance of colorectal CSCs since it is involved in the activation of the tyrosine kinase receptor c-Met[58]; CD166, a mesenchymal stem cell marker (see below), has been suggested as a potential co-CSCs marker, together with CD44, in human CRC, since in xenograft $CD44^+/CD166^+$ cells have a higher tumorigenicity as compared to $CD44^+CD166^-$ cells. The surface phenotype $EpCAM^{high}/CD44^+/CD166^+$ has been proposed as an alternative to the CD133 positivity for the selection of colon CSCs[18] and $CD44^+$ CRC cells have been shown to display a higher proliferation, more robust formation of colonies, less spontaneous apoptosis and a higher resistance to drug-induced cell death compared to $CD44^-$ cells[47].

More controversial are the findings regarding the role of CD44 in tumor progression and in the development of metastases in CRC. Several studies showed that expression of CD44 on tumor cells is correlated with

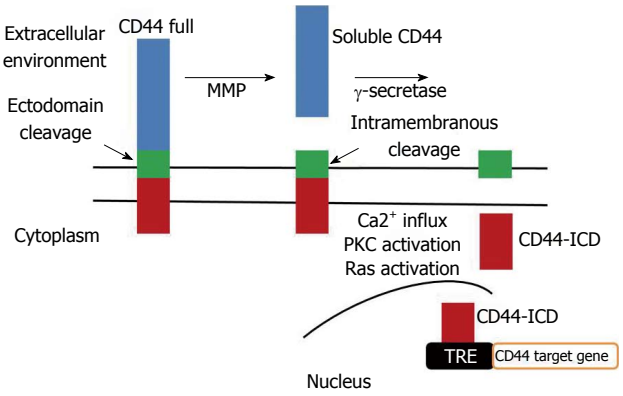


Figure 10 Schematic representation of CD44 sequential proteolytic processing. CD44 undergoes sequential proteolytic cleavages in the ectodomain and intramembranous domain. Cleavage of CD44 ectodomain generates soluble CD44 that regulates cell attachment and migration and induces the intramembranous domain cleavage, releasing the intracellular domain of CD44 (CD44-ICD). CD44-ICD translocates to the nucleus, where it activates gene transcription, including CD44 itself, via binding to TPA-responsive elements (TRE). MMP: matrix metalloproteinase.

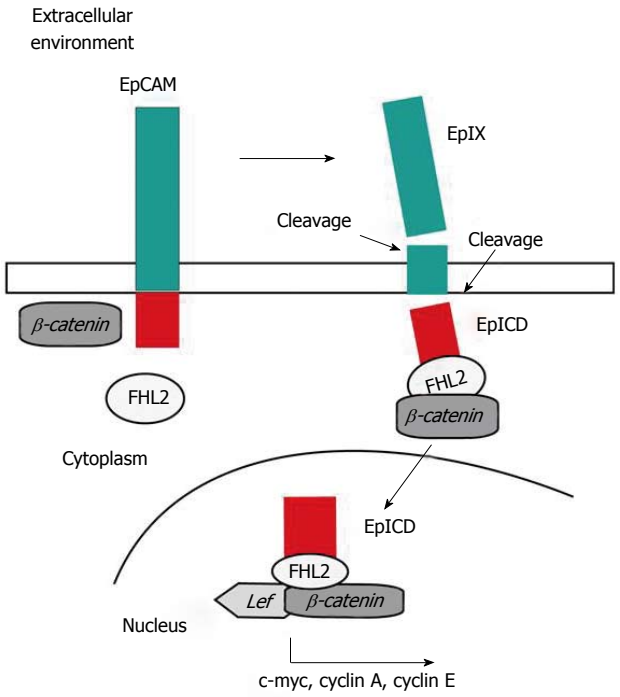


Figure 11 Schematic representation of epithelial cell adhesion molecule activation. Cleavage of full length epithelial cell adhesion molecule (EpCAM) generates EpEx (extracellular domain of EPCAM) and EpICD (EpCAM Intracellular Domain) fragments. EpICD binds the scaffold protein FHL2 and joins to the transcriptional regulators β-catenin and, within the nucleus, interacts with LEF, binds to DNA and induces gene activation.

tumor progression and metastasis while others have suggested an inverse correlation or no correlation at all[57,58].

Down-regulation of CD44 was initially related to a decrease in the metastatic potential of CRC cells[61], while more recently Dallas reported that down-regulation of CD44 leads to an increase of the metastatic and migratory potential of CRC cells[62]. It was observed that high-grade CRC have higher CD44 expression levels compared to low-grade tumors and this over-expression was associated with a reduced patients survival[63]. On the other hand, Ylagan *et al*[64] reported that the loss, rather than an increased expression, of CD44 is associated with an increased tumor aggressiveness while Fernández *et al*[65] demonstrated that CD44 expression levels were related to proliferation in CRC, but not with patients outcome. Subsequently, CD44 expression in human CRC was associated with the depth of invasion and lymph node involvement, and CD44s overexpression was suggested to be an independent unfavorable prognostic factor for overall survival in advanced CRC[66]. These findings were not confirmed by Lugli *et al*[67] who reported that the loss of CD44 is associated with more advanced tumor stage, the presence of vascular invasion, lymph node involvement and an infiltrating tumor border. Patients with tumors displaying a loss of CD44 or CD166 expression in the invasive front of the lesion had an adverse outcome compared with those expressing at least one of the two markers[67] (Table 3).

Further studies are warranted to further understand the suitability of CD44 molecule as a CSC marker in CRC and its role in human colorectal tumorigenesis.

EpCAM

Epithelial cell adhesion molecule (EpCAM), initially described in 1979 as a tumor associated antigen in human CRC[68], is a 30-40 kDa transmembrane glycoprotein showing frequent and high-level expression in a variety of human epithelial normal and cancer tissues, including colon[69]. It has been also detected on normal stem and progenitor cells and in cancer-initiating cells isolated from colon, breast, pancreas and prostate carcinomas[16,17,70]. Several evidences demonstrate that EpCAM is involved in cell adhesion, proliferation, differentiation and migration as well as in cancer and stem cells signaling[71,72].

The human EpCAM protein was independently identified by various research groups and, for this reason, several terms have been used to identify the molecule on the basis of the monoclonal antibody used to identify it[70]. However, it has been lately agreed the use of the term "EpCAM", without other specifications[70].

EpCAM displays a marked expression gradient from crypts to the apex of villi in normal colon tissue: adenoma development is associated with an increased EpCAM expression, and EpCAM overexpression is frequently observed in colorectal carcinoma[73]. Denzel *et al*[72] demonstrated that EpCAM is less accessible to antibodies in colon adenomas than in cancer because, in the last condition, EpCAM is activated by proteolysis in EpICD, the intracellular domain of EpCAM, and is intracellularly redistributed in dispersed patterns. They also showed that EpICD translocates into the cytoplasm together with the scaffold protein FHL2 and joins to the transcriptional regulator β-catenin to form a complex which, within the nucleus, interacts with Lef and binds to DNA inducing c-myc, cyclin A and cyclin E expression[72] (Figure 11). These findings were further confirmed by the ob-

servation that nuclear and cytoplasmic EpICD in solid epithelial cancers, such as colon, are increased, while the expression of membrane EpEx, the extracellular domain of EPCAM, is absent or reduced[74].

EpCAM was initially identified as a marker of human colorectal CSCs by Dalerba *et al*[18] who focused on two markers previously identified on human breast CSC: CD44 and EpCAM. Two main populations of epithelial cells were sorted from primary human CRCs by FACS: $EpCAM^{high}/CD44^{+}$ and $EpCAM^{low}/CD44^{-}$ and their tumorigenic properties were assessed. The results obtained demonstrated that the injection of 200 to 500 $EpCAM^{high}/CD44^{+}$ cells in NOD/SCID mice were sufficient to give rise to a tumor, whereas up to 104$EpCAM^{low}/CD44^{-}$ cells failed to form visible tumors. The xenograft tumors from $EpCAM^{high}/CD44^{+}$ reproduced the histopathology and phenotypic heterogeneity of the original tumors including the presence of variable percentages of both $EpCAM^{high}/CD44^{+}$ and $EpCAM^{low}/CD44^{-}$ cell populations[18]. They also verified that human $EpCAM^{high}/CD44^{+}$ cells from xenogenic colorectal tumors can be further stratified on the basis of the expression of the protein surface marker CD166, which could be used for the enrichment of colorectal CSCs[18]. Similar conclusions were also reached by Dylla *et al*[75] who suggested that one of the possible reasons of CRC resistance to chemotherapeutic agents might be at least in part attributed to the presence of $EpCAM^{+}/CD44^{+}$ CSC since residual tumors after chemotherapy are enriched of these cells.

All these findings about EpCAM signaling and its involvement in various cellular processes, provide a strong basis for further studies to better understand its potential clinical, prognostic and therapeutic value in CRC patients.

CD24

CD24 is a small, heavily glycosylated mucin-like adhesion molecule consisting of 27 amino acids with several potential O- or N-linked glycosylation sites, which lead to a molecular mass ranging between 38 and 70 kDa[76]. CD24 is attached to cell membranes by a phosphatidylinositol anchor and is expressed physiologically in the developing pancreas and brain and in pre-B lymphocytes, in regenerating muscle, in normal keratinocytes and in renal tubules[76]. It is physiologically localized in lipid rafts where it seems to be involved in the regulation of cell adhesion and signaling[76].

CD24 is expressed in various hematologic malignancies and solid tumors such as neuroblastoma, rhabdomyosarcoma, renal cell carcinoma, breast, ovarian, prostate, lung, colorectal and gastric cancer[76,77]. The observations that CD24 is one of the possible ligands of P-selectin and one of the adhesion receptors expressed by activated endothelial cells and platelets suggest that this molecule might play a role in the process of cancer metastasis[76].

Nestl *et al*[77] initially reported an increased expression of CD24 RNA in CRC: they showed that CD24 mRNA was weakly detectable in normal colonic mucosa but highly expressed in tumor cells, and to a lesser extent in the surrounding stroma. Later, Weichert *et al*[78] analyzed CD24 protein expression in colon cancer cell lines and human CRC and correlated it to clinic-pathological variables including patient survival. From this study emerged that the majority of CRCs showed both membranous and cytoplasmic CD24 staining, and that the membranous CD24 staining was associated with metastasis but was not significantly related to other clinic-pathological variables, while the cytoplasmic staining could be considered an independent prognostic marker related with a poor patient survival[78]. Conversely, Sagiv *et al*[19] failed to demonstrate any prognostic significance of CD24 expression level in CRC: in their study CD24 was similarly highly expressed in both adenomas and carcinomas. Moreover, unlike Weichert findings, they only reported a membranous staining. The same study also showed that CD24 is expressed early in the multistep process of CRC carcinogenesis, a finding consistent with its potential role as CSC marker.

Contradictory data have been reported in the literature concerning the prognostic value of CD24 whose expression levels have been reported to be not related with survival of CRC patients despite their significant relationship with conventional clinic-pathological factors such as tumor invasiveness and degree of differentiation[79]. Therefore, the real prognostic role of CD24 in CRC remains still unclear and controversial and it should be better elucidated by further studies.

Spheroid cultures of primary CRC have tumor-initiating capacity and are capable of inducing tumors upon xenotransplantation. These tumors resemble the original neoplasms both from a morphological point of view and the expression of specific markers[16]. Vermeulen *et al*[14] suggested that the co-expression of CD133 and CD24 could improve the identification of the clonogenic population within the spheroid cultures, and that both markers are downregulated during cell differentiation. CD24 was also used, in association with CD44, to identify and characterize CSCs from CRC cell lines by Yeung *et al*[80]. They demonstrated that the $CD44^{+}/CD24^{+}$ subpopulation of cells, isolated using FACS sorting, was the most clonogenic, giving rise to the highest proportion of megacolonies (complex structures resembling colonic crypts) compared to $CD44^{-}/CD24^{-}$ cells. $CD24^{+}$ subpopulation was also shown to exhibit cancer stem-like properties such as enhanced chemotherapy-resistance, self-renewal and tumorigenic capacity both *in vitro* and *in vivo*, compared to $CD24^{-}$ subpopulation isolated from CRC cell lines[80].

To our knowledge only few studies have investigated the underlying molecular mechanisms and the exact role played by this cell surface marker in CRC tumorigenesis. Thus, CD24 has been shown to activate Erk1/2 and p38 MAPKs and to increase the activity of Src and induce miR-21 expression, which in turn inhibits the expression of Pdcd4 and PTEN. On the other hand, the expression of CD24 and Src appears to be suppressed by miR34a through the downregulation of miR21[81].

Further studies are warranted to clarify the real activity of CD24 in CSCs and the key regulatory molecular

networks involved in its role in colorectal tumorigenesis.

CD29

CD29 (β1-integrin) is a member of the integrin family and consists of a large extracellular domain, a single transmembrane stretch and a short cytoplasmic domain. It acts as a receptor for extracellular matrix proteins and activates signaling molecules and pathways that regulate cell migration, proliferation, survival, differentiation and death[82].

In fact, CD29, by binding with fibronectin or Type I collagen, allows activation of Fak by Src leading to the activation of Erk that regulates cell proliferation. Erk, through phosphorylation of myosin light chain (MLC) by MLC kinase, also regulates cytoskeleton reorganization and cell motility. Moreover, CD29 regulates cell survival through the activation of Akt pathway[83].

CD29 has been initially described as an epidermal stem cell marker, and subsequently as a regulator of spermatogonial stem cells homing and of hematopoietic stem cells[22]. In normal human colon, CD29 is expressed at the bottom of the crypts, where it identifies a cell population that is capable of forming colonies in agar. For this reason, CD29 has been proposed as a stem/progenitor cell marker[27] and as a marker of colon CSCs. In fact, it has been shown that $CD133^+CD29^+$ colon CSCs are biologically characterized by self-renewal, proliferation and differentiation[14,20].

CD29, with E-cadherin, mediates cell-cell and cell-collagen interactions that are required for the maintenance of the differentiated phenotype of human CRC cells. Thus, CD29 downregulation may be responsible of the switch from differentiated to undifferentiated phenotype *in vivo*[84]. CD29 seems to be also implicated in the enhancement of the metastatic activity of CRC cells. In fact, Okazaki *et al*[85] showed that CD29 was significantly increased *in vivo* in metastases derived from human CRC cells. CD29 expression appears also to increase in the passage from adenoma to adenocarcinoma and with increasing tumor stage[86].

CD29 expression may be also associated with overall survival in CRC patients. In fact, loss of CD29 expression is associated with advanced stage and with poor prognosis and CD29 expression decreases in metastatic lesions[87], although other Authors have suggested that CD29, in combination with CD49b, might contribute to the acquisition of a metastatic potential in CRC cells. Finally, CD29 expression has been shown to identify the population of CRC cells that are more resistant to radio and chemo-therapy[88]. Further studies are needed to understand the specific role of CD29 as CSC marker as well as in the progression of CRC.

Lgr5

Lgr5, (Leucine-rich repeat-containing G protein-coupled receptor 5) also known as Gpr49, is an orphan G protein coupled receptor, characterized by a large leucine-rich extracellular domain and seven transmembrane domains. It is a receptor for R-spondin proteins which represent secreted agonists of the canonical Wnt/β-catenin signaling pathway[89,90].

Lgr5 is a member of the glycoprotein hormone receptor subfamily that includes the thyroid-stimulating, the follicle-stimulating and the luteinizing hormones receptors[21].

Lgr5 was first identified in human colon cancer cell lines harboring Wnt activating mutations as a Wnt target gene[4,91] and was then shown to be overexpressed in other human malignancies such as ovarian, hepatocellular, esophageal and basal cell carcinomas[92].

Since Lgr5 is one of Wnt target genes, it is not surprising that this protein is found expressed in different stem cells[5,93]. In the intestine Lgr5 is expressed in mature intestinal stem cells at the bottom crypt[4,5]; more specifically, Barker *et al*[4], using in situ hybridization demonstrated that Lgr5 is selectively expressed on few proliferating cells alternated with Paneth cells at the bottom of the crypts in the small intestine. These cells, known as crypt base columnar cells, are cycling cells and represent intestinal stem cells. These findings have suggested that Lgr5 could have an important role in colorectal carcinogenesis and that it could be an ideal marker of colorectal CSCs.

Several research groups have investigated whether Lgr5 could play a role in colorectal tumorigenesis and several studies suggested that there is a close correlation between Lgr5 expression and colon cancer progression[90,92].

In fact, Lgr5, which is normally localized to the basal intestinal crypt area, is expressed only in the peripheral region of adenomas and ubiquitously in established adenocarcinomas. It has been hypothesized that the accumulation of genome mutations occurring during the process of malignant transformation, might lead to loss of $Lgr5^+$ cells polarity that can thus migrate to the tumor-host interface (carcinoma *in situ*) and then in all the tumor (advanced cancer)[94]. The selective expression of Lgr5 in the peripheral region of adenomas supports the hypothesis that it might mark intestinal CSCs. In favor of this hypothesis is the work of Batlle *et al*[95] that reported that Lgr5 is selectively expressed on human colon CSCs. This finding has been further confirmed by Kemper[38] who demonstrated that Lgr5 identifies the CSC fraction in CRC and that it is expressed at high levels in spheroid cultures derived from primary CRC (that are known to be enriched for CSCs) and decreased following cellular differentiation[38].

Since Lgr5 is expressed at high levels in both colorectal adenomas and adenocarcinomas it likely plays an important role not only in the early but also in the late events of tumorigenesis, such as invasion and metastasis. Moreover, high Lgr5 expression has been shown to correlate with mesenchymal characteristics of tumors, such as high expression of vimentin and low expression of miR-200c, and with increased invasiveness and lymph node metastases[92,94].

Overall, the available evidence suggests that Lgr5 could play a key role in the development and progression

of CRC and might represent a useful marker to identify and/or target CSC in colon cancer.

CD166

Activated leukocyte cell adhesion molecule (ALCAM), also known as CD166, is a member of a subgroup of transmembrane glycoproteins in the immunoglobulin superfamily, characterized by the presence of five extracellular immunoglobulin-like domains (VVC2C2C2)[96].

CD166 is able to form low-affinity hemophilic interactions and much stronger heterophilic interactions with CD6 expressed on T lymphocytes, thymocytes and on a subset of B cells[97].

Beside hematopoietic cells, expression of CD166 has been reported in a wide variety of tissues and cells including selected epithelia, lymphoid and myeloid cells, fibroblasts, neurons, hepatocytes, pancreas acinar and islet cells[98]. CD166 is also present in a large number of tumors including breast, lung, colon and prostate cancer and melanoma[98].

In the small intestine and in the colon, CD166 was observed at high levels on the surface of cells within the stem cell niche at the base of the crypt, but little is known about its endogenous function. However, CD166 seems to be involved in the morphogenesis of tubular structures by cell-cell and cell-matrix interactions[99].

Expression of CD166 in colon cancer has been analyzed by several groups with conflicting results. Weichert *et al*[100] suggested that CD166 up-regulation is an early event in colon tumorigenesis because it was found in all adenomas of the colon. Moreover, they reported by immunohistochemistry both a cytoplasmic and membranous staining for CD166 in CRC and a correlation between high membranous CD166 expression and poor prognosis. On the contrary, Horst and collaborators did not find any correlation between CD166 expression and CRC patients outcome[101].

The study conducted by Lugli revealed an association between the loss of CD166 and an increase in tumor size, lymph node metastasis, tumor infiltration and a shorter overall survival[67].

These findings have been partially confirmed by a recent work showing that CD166 expression is a positive prognostic marker for overall survival in CRC patients. CD166 expression in well differentiated CRC suggests a role of the protein in the early stages of tumorigenesis and, since CD166 seems to be involved in cell-cell and cell-matrix adhesion, its loss may be associated with reduced cell adhesion and therefore with a higher metastatic potential of tumors[102].

It has been recently suggested that CD166 may contribute to the identification of colorectal CSCs[18] but its role in CRC tumorigenesis as well as a marker of CSC remains to be defined.

THERAPEUTICS RELEVANCE OF COLORECTAL CANCER STEM CELLS

Cancer stem cells are believed to be resistant to chemo- and radiation-therapy and are indicated to be the cause of cancer relapse and metastasis: conventional anticancer therapy wipes out the bulk populations but the surviving CSCs repopulate the tumor (Figure 3). Therefore, targeting both CSC and the bulk populations is essential for complete tumor eradication. Thus, the identification of colorectal CSC markers and their signaling pathway is crucial for the development of novel therapies which could specifically target these cells.

The potential therapeutic strategies aimed at selectively target CSCs, which are beginning to be experimentally validated, include the elimination of CSCs through agents which target specific markers of CSC (such as monoclonal antibodies) or interfere with CSC-specific pathways[103].

Todaro *et al*[104] demonstrated that $CD133^+$ colon CSC produce and use the cytokine IL-4 to protect themselves from apoptosis caused by conventional chemotherapy agents, 5-fluorouracil and oxaliplatin. In fact, the simultaneous treatment with antibodies to IL-4 greatly increased the antitumoral cytotoxic activity of the drugs.

It has been also reported that a 5-fluorouracil and oxaliplatin chemoresistant derivative of the HT29 human CRC cell line displayed an enrichment of $CD133^+$ and $CD44^+$ cells with an increased expression of the Type 1 insulin-like growth factor receptor (IGF-IR). Treatment with a monoclonal antibody to IGF-IR induced a significant inhibition of tumor growth, thus demonstrating an enhanced sensitivity of colon CSC to IGF-IR specific targeted therapy[105].

More recently, Bach *et al*[106] used measles viruses, oncolytically active against various types of human cancer, to generate CD133-specific measles viruses (MV) and to provide a new CSC-specific anticancer therapy. They were able to efficiently infect the primary colon spheres to test the oncolytic activity of CD133-MV on colon primary tumor cells. The infection caused a rapid loss of $CD133^+$ cells and, when implanted in NSG mice, the CD133-MV infected tumor spheres formed tumors smaller than uninfected tumor spheres. However, no effect in term of tumor volume was observed when the resected tumors were transplanted in secondary mice and the re-isolated tumors contained 70% of $CD133^+$ cells[106].

Given that CD133 is also expressed on normal stem cells, Bostad *et al*[107] have developed a site-specific strategy that allows to release the drug only in the tumor area. They developed an immunotoxin targeting CD133 by using the photochemical internalization (PCI) technology. The biotinylated anti-CD133 antibodies were mixed with streptavidin-saporin (sap) to form the model of anti-CD133-sap immunotoxin. Saporin, a plant toxin, is a potent ribosome inactivating protein and was used as the toxin component of the immunotoxin. The aim of this technology was to avoid the degradation of the drug by the lysosomes before the drug has interacted with its biological target, and the main advantage should be the accumulation of the photosensitizer preferably in the neoplastic tissue. This report demonstrated that the $CD133^{high}$ population of WiDr colon cancer cells is more resistant

to photodynamic therapy than the $CD133^{low}$ population but the PCI of a CD133-targeting toxin is able to sensitize and destroy these resistant cells. Thus, PCI-based anti-CSC strategy could be a specific method for a selective killing of $CD133^{+}$ CSCs while sparing normal stem cells[107]. Chen *et al*[108] tested the effects of CD133 monoclonal antibody (Miltenyi) on hepatocarcinoma cells. The CD133 monoclonal antibody treatment, under extracellular low glucose condition, inhibited the proliferation of hepatocarcinoma cells, suppressed spheroid and colony formation, attenuated xenograft tumors and improved the efficiency of chemotherapy. Moreover, Swaminhatan and others developed nanoparticles formulated using the biodegradable poly (D, Llactide- co-glycolide) polymer and surface functionalized with an anti-CD133 antibody (CD133NPs). The CD133NPs were loaded with paclitaxel and were able to reduce the fraction of tumor-initiating cells *in vitro* and tumor recurrence in the MDA-MB-231 xenograft tumor model[109].

EpCAM has been also suggested as a potential target for the development of a CSC-specific therapy for CRC. Several clinical trials have already evaluated the efficacy of a monoclonal antibody to EpCAM for a targeted treatment of CRC. Edrecolomab, a murine monoclonal anti-EpCAM antibody, was the first immunotherapeutic agent licensed for the use in a large-scale human anti-tumor immunotherapy trial. In 1994, Riethmüller *et al*[110] randomly assigned to adjuvant therapy with Edrecolomab a series of patients with a resected Dukes' C CRC: they showed an improved survival rate, and a reduction of mortality and disease recurrence[110,111]. These promising results were not further confirmed. In fact, Punt *et al*[111] showed that the addition of Edrecolomab to fluorouracil and folinic acid in the adjuvant treatment of resected stage Ⅲ CRC did not provide any further improvement in term of survival, and that the immunotherapy alone was associated with a significant shorter disease-free survival[111]. Similar findings have been reported by Fields *et al*[112] who adopted a combination of fluorouracil-based therapy and Edrecolomab for the treatment of stage Ⅲ colon cancer patients, getting poor results.

More recently, Waldron *et al*[113] have characterized a biospecific target toxin, which is composed by anti-EpCAM and anti-CD133 scFv (single-chain variable fragment), and have focused on three different types of carcinoma: head and neck, breast, and colon carcinoma. The toxin, called deimmunized CD133KDEL (dCD-133KDEL), was synthesized using an anti-CD133 scFv that recognized the loop two of the extracellular domain of CD133 and both the glycosylated and unglycosylated forms of CD133. The anti-CD133 scFv was fused with an anti-EpCAMscFv and with a truncated form of Pseudomonas exotossin A (PE38) and the construct called dEpCAMCD133KDEL and showed a strong inhibition of proliferation in CRC cell lines.

CD44 can represent a suitable therapeutic target for CRC, since it presents two distinct forms between normal and cancer cells. In fact, the different local environmental pressures are responsible for different splicing and post-translational modifications which give rise to different CD44 molecules that can be recognized by specific agents useful for both diagnosis and therapy[114].

CD44 knockdown was shown to inhibit tumor growth and metastasis *in vivo*[61], a finding confirmed by Du *et al*[58] who used lentiviral RNA interference to stably knock down CD44 or CD133 in CRC primary cells isolated from patients. These Authors reported that knockdown of CD44 reduced clonal formation, whereas CD133 knockdown had little effect compared to control.

The combination of curcumin and dasatinib has been also suggested as a therapeutic strategy for chemo-resistant CRC. In fact, the combination therapy with curcumin and dasatinib inhibited the growth of chemo-resistant HT29 and HCT-116 CRC cells, the formation of colonospheres and extracellular invasion. The expression of CSC markers CD133, CD44, CD166 and ALDH1 displayed a 25%-30% decrease in cells treated with curcumin and dasatinib thus suggesting that the combination may be used as a specific CSC targeted therapy to prevent recurrence of CRC[115].

Another study demostrated that difluorinated-curcumin in combination with 5-fluorouracil and oxaliplatin, the standard of CRC chemotherapy, was more potent than curcumin in reducing CD44 and CD166 expression in chemo-resistant CRC cells. This effect was associated with growth inhibition, induction of apoptosis and disintegration of colonospheres[116].

Misra *et al*[114] demonstrated that CD44v6 knockdown reduced the ability of CRC cells to signal through hyaluronan-CD44v6. They encapsulated plasmidic DNA coding CD44v6 shRNA into transferrin (Tf)coated nanoparticles which are recognized by Tf-receptor (TF-R) present at high level on tumor cells which then internalize the particles by receptor-mediated endocytosis. These nanoparticles were delivered within pre-neoplastic and neoplastic colon tissues in the Apc Min/mice model, causing inhibition of the CD44v6 expression. This inhibition was associated with a reduced adenoma number and growth through a hyaluronan/CD44v6/ErB2/Cox 2 interaction pathway[114].

Mesoporous silica nanoparticles (MSNs) have been proposed as nanocarriers for several anticancer treatments. Yu *et al*[117] have developed a targeted drug delivery system based on hyaluronic acid (HA) modified MSNs (HA-MSNs). HA-MSNs have a specific affinity to CD44 overexpressed on CRC cells and can enter cells *via* the HA receptor mediated endocytosis pathway. Doxorubicin (Dox), an anticancer drug, has been encapsulated into MSNs with or without HA. HCT-116 cells were treated with free Dox, Dox-HA-MSNs or Dox-MSNs. The cells treated with Dox-HA-MSNs presented a stronger inhibition of proliferation (51%) compared to the other two groups[117].

A new potential therapeutic approach targeting CSCs was suggested by Sagiv *et al*[118] who showed that the growth of human CRC cells lines, expressing the presumptive CSC marker CD24, was inhibited after treatment with three different anti-CD24 monoclonal

antibody which showed a synergistic effect with chemotherapeutic agents[19,118].

Downregulation of CD29 expression by antisense oligonucleotide in the HT-29 human CRC cell line reduced both tumor cells migration *in vitro* and hepatic metastasis *in vivo*[37]. Interestingly, barberine, a botanical alkaloid with cytotoxic effects on most type of cancer cells, can inhibit the migration of SW480 and HCT116 CRC cells through a decrease of CD29 expression level *via* AMP-activated protein kinase thus suggesting the possibility to use this drug to specifically target CD29-expressing CSCs[119].

Targeting of CSC can be also obtained by interfering with the pathways involved in stemness maintenance such as Wnt, Hedgehog and Notch. Several approaches for CRC therapy have focused on Wnt pathway inhibition. Wnt family proteins are secreted intercellular signaling molecules that act as ligands to activate a specific signal transduction pathway. Upon binding of Wnt to its receptor Frizzled (FZD), the protein Disheveled (Dvl/Dsh) is activated and inhibits the Glycogen synthase kinase 3 (GSK-3) activity. The latter binds to axin, APC and Casein Kinase 1 (CK1), and forms a complex that binds β-catenin and promotes its degradation. When Wnt signaling inhibits GSK-3, β-catenin dissociates from the complex and enters the nucleus, where it binds to the DNA binding protein Tcf/Lef, becoming a transcription factor[120]. Efforts have been mainly devoted to the identification of small molecules inhibiting this pathway.

Chen *et al*[121], in a screen of a synthetic chemical library, identified a new small molecule able to inhibit the Wnt signaling. This compound, called IWP (inhibitor of Wnt production), inhibits the activity of Porcupine, a membrane-bound acetyltransferase, essential for Wnt production[121]. Similarly, using report-based screening approaches, Huang *et al*[122] found a small molecule, XAV939, that inhibits Wnt through the tankyrase inhibition, an event leading to an increase in the stability of axin and subsequent β-catenin degradation[122].

Pyrvinium, another small molecule promoting the degradation of β-catenin was identified by Thorne *et al*[123]. This molecule, promotes β-catenin phosphorylation through casein kinase activation. It was shown that pyrvinium treatment of CRC cells bearing APC or β-catenin mutation inhibits both Wnt signaling and proliferation[123].

Several studies have focused on the identification of molecules capable of destroying the interaction between Tcf/Lef and β-catenin and therefore of inhibiting α-catenin-dependent transcription. Three of these compounds were shown to inhibit CRC cells growth both *in vitro* and *in vivo*[124].

Emami *et al*[125] developed a compound, ICG-001, which specifically inhibited a co-activator essential for Wnt pathway activation. Treatment of CRC cell lines bearing APC or β-catenin mutations with ICG-001 induced cell death in a dose-dependent fashion while not affecting normal epithelial cells. An analogue of this compound was recently approved for phase I clinical testing[126].

The Wnt pathway has been also successfully inhibited using a specific anti-Wnt monoclonal antibody, which inhibits the proliferation and induces apoptosis in CRC cells, even in those with downstream mutations[127]. Numerous groups have tried to inhibit the Wnt pathway by inhibiting the FZD receptor activity.

A member of FZD family, FZD7, results predominantly expressed in CRC cells and it is implicated in canonical Wnt signaling in cells with APC or CTNNB1 mutations. The use of specific siRNA to knockdown the expression of endogenous FZD7 has proved effective in reducing the metastatic potential of CRC cells[128]. Similar effects have been obtained using an antibody targeting FZD7[129]. Remarkable results have been also obtained using inhibitors of Delta-like ligand 4 (DLL4), an important component of the Notch pathway. Human CRC xenografts treated with an anti-DLL4 antibody in combination with irinotecan have showed a reduction of CSCs and of tumor growth whereas treatment with irinotecan alone increased the percentage of CSCs[130,131].

CONCLUSION

The CSC model of tumorigenesis postulates that tumors are not cellularly homogenous but display a hierarchical structure and contain a rare population of cells, the CSC, that display the same self-renewal and proliferative potentials as normal stem cells associated with the capacity to give rise to tumors (Figure 2). As previously described, mounting evidence suggests the existence of a CSC population in human CRC[16,17].

It has been hypothesized that CSC may derive from transformation of quiescent, normal long-term stem cells or could result from the de-differentiation of more mature cells[15-17]. In CRC, the first hypothesis is supported by the observation that normal and cancer stem cells share similar properties and surface markers (*i.e.*, CD133 and Lgr5). However, it cannot be excluded that CSC might derive from cells that, at some specific stages of differentiation, undergo malignant transformation acquiring new properties including stem-like features. This hypothesis might explain the different aggressiveness of tumors which might relate to the different differentiation degree of cells undergoing the transformation event(s) as evidenced by the different tumor grading[15] (Figure 12).

The ability to identify and isolate CSC is essential to fully characterize them and to understand the molecular mechanisms responsible for their establishment and their maintenance. As mentioned, several approaches have been used to identify and isolate CSC the most important being the antibody-based technologies targeting CSC-specific surface markers. However, different antibodies, techniques and protocols are used in different studies and these certainly contribute to the conflicting results present in the literature. Thus, it will be important to define standardized procedures and reagents to identify CSC in clinical samples. Moreover, several questions remain unresolved especially regarding the significance of CSC

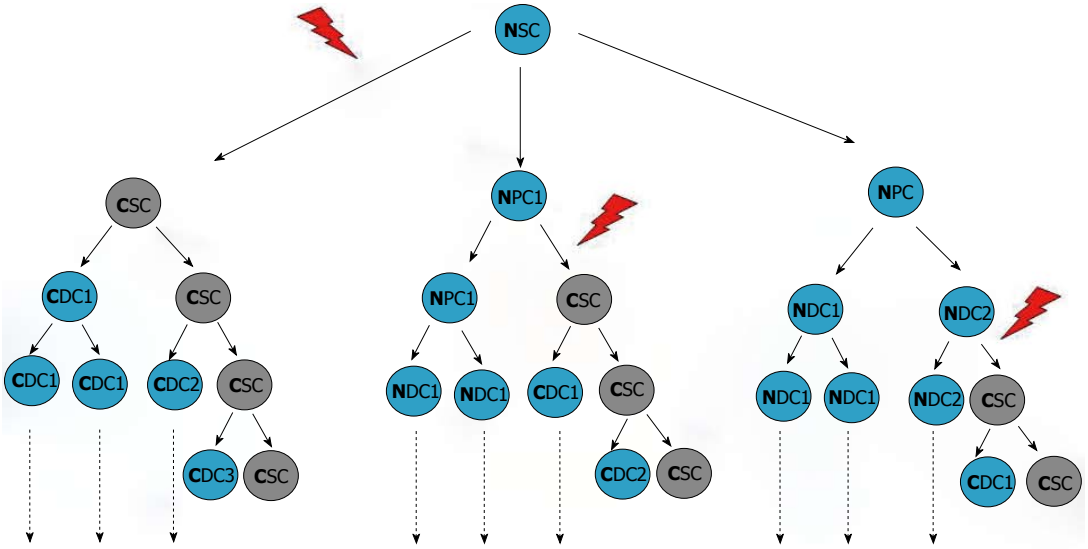


Figure 12 Origin of cancer stem cells and tumor heterogeneity. It has been hypothesized that CSCs may derive from transformation of quiescent, normal stem cells (left) or could result from the de-differentiation of more mature cells which might re-acquire the capability of self-renewal. Other mutations might occur following transformation in both cases. SC: Stem cell; PC: Progenitor cell; DC: Differentiated cell; **C**: Cancer; **N**: Normal; CSCs: Cancer stem cells.

markers and whether they play a direct role in essential CSC properties such as self-renewal and tumor-initiation ability or they are just markers of stem-like cells with no relevant physiological functions[44]. This is a very important issue in view of the possibility to develop specific anti-CSC therapies.

Indeed, the CSC model of tumorigenesis implies that targeting of CSC is essential for a complete eradication of the disease (Figure 3). This consideration fits well with the disappointing daily experience of oncologists facing occasional complete responses that do not translate into cure for patients. Indeed, the model hypothesizes that CSC must be completely eliminated in order to eradicate the disease and prevent recurrences/metastases. However, CSC have been reported to be relatively resistant to standard anticancer therapies, such as radiation and chemotherapy, which target rapidly proliferating cells[103].

Thus, initial responses to treatment could represent therapeutic effectiveness against the bulk cancer cells while sparing rare quiescent CSC which would then be responsible for tumor re-growth both at primary and metastatic sites. According to this model, a better understanding of the biology of CSC is essential to improve efficacy of anticancer therapies and several groups are pioneering the possibility of specifically targeting CSC through multiple approaches, as previously described[107,117,123]. A big issue will be the identification of substantial differences between normal and cancer stem cells and/or specific therapeutic strategies that would allow the development of drugs specifically targeting CSC while sparing normal counterparts. From this perspective, another important point to keep in mind is that standard response parameters might not be suitable to evaluate specific CSC-targeting therapies. Indeed, current evaluation criteria only take in consideration the effects of treatment on tumor bulk and thus might underestimate the effect of a therapy specifically targeting a rare population of cells within tumor mass. Thus, it will likely be important to re-examine the standard criteria to evaluate response therapy and other approaches, such as new CSC-specific imaging techniques, might be needed to this aim. This does not mean that conventional therapies will no longer have a place in the future anti-cancer protocols despite the fact that CSC may be resistant to them. Indeed, it seems realistic to anticipate that a useful approach to improve current treatment of solid tumors, including CRC, will be the combination of a specific anti-CSC treatment with traditional agents (*i.e.*, 5-fluorouracil and/or oxaliplatin) that can debulk the mass of cancer cells.

In conclusion, the CSC model of tumorigenesis has the potential to radically revolutionize the way how we look at malignant diseases as well as the clinical management of CRC patients. To this aim, it will be essential a definitive assessment of the roles that putative CSCs play in the development of human CRC and in specific aspects of malignancy. The ultimate proof of the relevance of CSCs in tumor development and in the clinical management of CRC cancer patients will be the demonstration that specific targeting of CSCs can improve patients outcomes, a goal strongly awaited by scientists, oncologists and, especially, patients.

REFERENCES

1 **Ferlay J**, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JW, Comber H, Forman D, Bray F. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. *Eur J Cancer* 2013; **49**: 1374-1403 [PMID: 23485231 DOI: 10.1016/j.ejca.2012.12.027]

2 **McDonald SA**, Preston SL, Lovell MJ, Wright NA, Jankowski JA. Mechanisms of disease: from stem cells to colorectal cancer. *Nat Clin Pract Gastroenterol Hepatol* 2006; **3**: 267-274 [PMID: 16673006 DOI: 10.1038/ncpgasthep0473]

3 **Blanpain C**, Horsley V, Fuchs E. Epithelial stem cells: turn-

ing over new leaves. *Cell* 2007; **128**: 445-458 [PMID: 17289566 DOI: 10.1016/j.cell.2007.01.014]
4 **Barker N**, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ, Clevers H. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 2007; **449**: 1003-1007 [PMID: 17934449 DOI: 10.1038/nature06196]
5 **Sangiorgi E**, Capecchi MR. Bmi1 is expressed in vivo in intestinal stem cells. *Nat Genet* 2008; **40**: 915-920 [PMID: 18536716 DOI: 10.1038/ng.165]
6 **Moore KA**, Lemischka IR. Stem cells and their niches. *Science* 2006; **311**: 1880-1885 [PMID: 16574858 DOI: 10.1126/science.1110542]
7 **Medema JP**, Vermeulen L. Microenvironmental regulation of stem cells in intestinal homeostasis and cancer. *Nature* 2011; **474**: 318-326 [PMID: 21677748 DOI: 10.1038/nature10212]
8 **Fearon ER**, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; **61**: 759-767 [PMID: 2188735 DOI: 10.1016/0092-8674(90)90186-I]
9 **Pinto D**, Gregorieff A, Begthel H, Clevers H. Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. *Genes Dev* 2003; **17**: 1709-1713 [PMID: 12865297 DOI: 10.1101/gad.267103Genes]
10 **Korinek V**, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, Vogelstein B, Clevers H. Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. *Science* 1997; **275**: 1784-1787 [PMID: 9065401 DOI: 10.1126/science.275.5307.1784]
11 **Haramis AP**, Begthel H, van den Born M, van Es J, Jonkheer S, Offerhaus GJ, Clevers H. De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine. *Science* 2004; **303**: 1684-1686 [PMID: 15017003 DOI: 10.1126/science.1093587]
12 **He XC**, Zhang J, Tong WG, Tawfik O, Ross J, Scoville DH, Tian Q, Zeng X, He X, Wiedemann LM, Mishina Y, Li L. BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. *Nat Genet* 2004; **36**: 1117-1121 [PMID: 15378062 DOI: 10.1038/ng1430]
13 **Dexter DL**, Spremulli EN, Fligiel Z, Barbosa JA, Vogel R, VanVoorhees A, Calabresi P. Heterogeneity of cancer cells from a single human colon carcinoma. *Am J Med* 1981; **71**: 949-956 [PMID: 7315857 DOI: 10.1016/0002-9343(81)90312-0]
14 **Vermeulen L**, Todaro M, de Sousa Mello F, Sprick MR, Kemper K, Perez Alea M, Richel DJ, Stassi G, Medema JP. Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity. *Proc Natl Acad Sci USA* 2008; **105**: 13427-13432 [PMID: 18765800 DOI: 10.1073/pnas.0805706105]
15 **Gangemi R**, Paleari L, Orengo AM, Cesario A, Chessa L, Ferrini S, Russo P. Cancer stem cells: a new paradigm for understanding tumor growth and progression and drug resistance. *Curr Med Chem* 2009; **16**: 1688-1703 [PMID: 19442140 DOI: 10.2174/092986709788186147]
16 **Ricci-Vitiani L**, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, De Maria R. Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007; **445**: 111-115 [PMID: 17122771 DOI: 10.1038/nature05384]
17 **O'Brien CA**, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 2007; **445**: 106-110 [PMID: 17122772 DOI: 10.1038/nature05372]
18 **Dalerba P**, Dylla SJ, Park IK, Liu R, Wang X, Cho RW, Hoey T, Gurney A, Huang EH, Simeone DM, Shelton AA, Parmiani G, Castelli C, Clarke MF. Phenotypic characterization of human colorectal cancer stem cells. *Proc Natl Acad Sci USA* 2007; **104**: 10158-10163 [PMID: 17548814 DOI: 10.1073/pnas.0703478104]
19 **Sagiv E**, Memeo L, Karin A, Kazanov D, Jacob-Hirsch J, Mansukhani M, Rechavi G, Hibshoosh H, Arber N. CD24 is a new oncogene, early at the multistep process of colorectal cancer carcinogenesis. *Gastroenterology* 2006; **131**: 630-639 [PMID: 16890615 DOI: 10.1053/j.gastro.2006.04.028]
20 **Fujimoto K**, Beauchamp RD, Whitehead RH. Identification and isolation of candidate human colonic clonogenic cells based on cell surface integrin expression. *Gastroenterology* 2002; **123**: 1941-1948 [PMID: 12454851 DOI: 10.1053/gast.2002.37065]
21 **Barker N**, Clevers H. Leucine-rich repeat-containing G-protein-coupled receptors as markers of adult stem cells. *Gastroenterology* 2010; **138**: 1681-1696 [PMID: 20417836 DOI: 10.1053/j.gastro.2010.03.002]
22 **Miraglia S**, Godfrey W, Yin AH, Atkins K, Warnke R, Holden JT, Bray RA, Waller EK, Buck DW. A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. *Blood* 1997; **90**: 5013-5021 [PMID: 9389721]
23 **Asahara T**, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997; **275**: 964-967 [PMID: 9020076 DOI: 10.1126/science.275.5302.964]
24 **Shaked Y**, Ciarrocchi A, Franco M, Lee CR, Man S, Cheung AM, Hicklin DJ, Chaplin D, Foster FS, Benezra R, Kerbel RS. Therapy-induced acute recruitment of circulating endothelial progenitor cells to tumors. *Science* 2006; **313**: 1785-1787 [PMID: 16990548 DOI: 10.1126/science.1127592]
25 **Grosse-Gehling P**, Fargeas CA, Dittfeld C, Garbe Y, Alison MR, Corbeil D, Kunz-Schughart LA. CD133 as a biomarker for putative cancer stem cells in solid tumours: limitations, problems and challenges. *J Pathol* 2013; **229**: 355-378 [PMID: 22899341 DOI: 10.1002/path.4086]
26 **Puglisi MA**, Sgambato A, Saulnier N, Rafanelli F, Barba M, Boninsegna A, Piscaglia AC, Lauritano C, Novi ML, Barbaro F, Rinninella E, Campanale C, Giuliante F, Nuzzo G, Alfieri S, Doglietto GB, Cittadini A, Gasbarrini A. Isolation and characterization of CD133+ cell population within human primary and metastatic colon cancer. *Eur Rev Med Pharmacol Sci* 2009; **13** Suppl 1: 55-62 [PMID: 19530513]
27 **Marzesco AM**, Janich P, Wilsch-Bräuninger M, Dubreuil V, Langenfeld K, Corbeil D, Huttner WB. Release of extracellular membrane particles carrying the stem cell marker prominin-1 (CD133) from neural progenitors and other epithelial cells. *J Cell Sci* 2005; **118**: 2849-2858 [PMID: 15976444 DOI: 10.1016/j.febslet.2009.01.048]
28 **Sgambato A**, Puglisi MA, Errico F, Rafanelli F, Boninsegna A, Rettino A, Genovese G, Coco C, Gasbarrini A, Cittadini A. Post-translational modulation of CD133 expression during sodium butyrate-induced differentiation of HT29 human colon cancer cells: implications for its detection. *J Cell Physiol* 2010; **224**: 234-241 [PMID: 20333645 DOI: 10.1002/jcp.22124]
29 **Shmelkov SV**, Butler JM, Hooper AT, Hormigo A, Kushner J, Milde T, St Clair R, Baljevic M, White I, Jin DK, Chadburn A, Murphy AJ, Valenzuela DM, Gale NW, Thurston G, Yancopoulos GD, D'Angelica M, Kemeny N, Lyden D, Rafii S. CD133 expression is not restricted to stem cells, and both CD133+ and CD133- metastatic colon cancer cells initiate tumors. *J Clin Invest* 2008; **118**: 2111-2120 [PMID: 18497886 DOI: 10.1172/JCI34401]
30 **Feng JM**, Miao ZH, Jiang Y, Chen Y, Li JX, Tong LJ, Zhang J, Huang YR, Ding J. Characterization of the conversion between CD133+ and CD133- cells in colon cancer SW620 cell line. *Cancer Biol Ther* 2012; **13**: 1396-1406 [PMID: 22954703 DOI: 10.4161/cbt.22000]
31 **Hsu CS**, Tung CY, Yang CY, Lin CH. Response to stress in early tumor colonization modulates switching of CD133-positive and CD133-negative subpopulations in a human metastatic colon cancer cell line, SW620. *PLoS One* 2013; **8**: e61133 [PMID: 23577199 DOI: 10.1371/journal.pone.0061133]
32 **Kojima M**, Ishii G, Atsumi N, Fujii S, Saito N, Ochiai A. Immunohistochemical detection of CD133 expression

in colorectal cancer: a clinicopathological study. *Cancer Sci* 2008; **99**: 1578-1583 [PMID: 18754869 DOI: 10.1111/j.1349-7006.2008.00849.x]
33 **Horst D**, Kriegl L, Engel J, Kirchner T, Jung A. CD133 expression is an independent prognostic marker for low survival in colorectal cancer. *Br J Cancer* 2008; **99**: 1285-1289 [PMID: 18781171 DOI: 10.1038/sj.bjc.6604664]
34 **Ong CW**, Kim LG, Kong HH, Low LY, Iacopetta B, Soong R, Salto-Tellez M. CD133 expression predicts for non-response to chemotherapy in colorectal cancer. *Mod Pathol* 2010; **23**: 450-457 [PMID: 20081809 DOI: 10.1038/modpathol.2009.181]
35 **Kawamoto A**, Tanaka K, Saigusa S, Toiyama Y, Morimoto Y, Fujikawa H, Iwata T, Matsushita K, Yokoe T, Yasuda H, Inoue Y, Miki C, Kusunoki M. Clinical significance of radiation-induced CD133 expression in residual rectal cancer cells after chemoradiotherapy. *Exp Ther Med* 2012; **3**: 403-409 [PMID: 22969903 DOI: 10.3892/etm.2011.438]
36 **Chao C**, Carmical JR, Ives KL, Wood TG, Aronson JF, Gomez GA, Djukom CD, Hellmich MR. CD133+ colon cancer cells are more interactive with the tumor microenvironment than CD133- cells. *Lab Invest* 2012; **92**: 420-436 [PMID: 22157717 DOI: 10.1038/labinvest.2011.185]
37 **Zhang SS**, Han ZP, Jing YY, Tao SF, Li TJ, Wang H, Wang Y, Li R, Yang Y, Zhao X, Xu XD, Yu ED, Rui YC, Liu HJ, Zhang L, Wei LX. CD133(+)CXCR4(+) colon cancer cells exhibit metastatic potential and predict poor prognosis of patients. *BMC Med* 2012; **10**: 85 [PMID: 22871210 DOI: 10.1186/1741-7015-10-85]
38 **Kemper K**, Versloot M, Cameron K, Colak S, de Sousa e Melo F, de Jong JH, Bleackley J, Vermeulen L, Versteeg R, Koster J, Medema JP. Mutations in the Ras-Raf Axis underlie the prognostic value of CD133 in colorectal cancer. *Clin Cancer Res* 2012; **18**: 3132-3141 [PMID: 22496204 DOI: 10.1158/1078-0432.CCR-11-3066]
39 **Mohammadi M**, Bzorek M, Bonde JH, Nielsen HJ, Holck S. The stem cell marker CD133 is highly expressed in sessile serrated adenoma and its borderline variant compared with hyperplastic polyp. *J Clin Pathol* 2013; **66**: 403-408 [PMID: 23436931 DOI: 10.1136/jclinpath-2012-201192]
40 **Coco C**, Zannoni GF, Caredda E, Sioletic S, Boninsegna A, Migaldi M, Rizzo G, Bonetti LR, Genovese G, Stigliano E, Cittadini A, Sgambato A. Increased expression of CD133 and reduced dystroglycan expression are strong predictors of poor outcome in colon cancer patients. *J Exp Clin Cancer Res* 2012; **31**: 71 [PMID: 22964035 DOI: 10.1186/1756-9966-31-71]
41 **Sgambato A**, Errico F, Caredda E, Puglisi MA, Cittadini A. Divergent expression of CD133 in different studies: the need for a consensus panel? *Int J Cancer* 2011; **128**: 2247-2249 [PMID: 20626045 DOI: 10.1002/ijc.25551]
42 **Zhu L**, Gibson P, Currle DS, Tong Y, Richardson RJ, Bayazitov IT, Poppleton H, Zakharenko S, Ellison DW, Gilbertson RJ. Prominin 1 marks intestinal stem cells that are susceptible to neoplastic transformation. *Nature* 2009; **457**: 603-607 [PMID: 19092805 DOI: 10.1038/nature07589]
43 **Arena V**, Caredda E, Cufino V, Stigliano E, Scaldaferri F, Gasbarrini A, Cittadini A, Sgambato A. Differential CD133 expression pattern during mouse colon tumorigenesis. *Anticancer Res* 2011; **31**: 4273-4275 [PMID: 22199291]
44 **Sgambato A**, Corbi M, Svelto M, Caredda E, Cittadini A. New Insights into the CD133 (Prominin-1) Expression in Mouse and Human Colon Cancer Cells. *Adv Exp Med Biol* 2013; **777**: 145-166 [PMID: 23161081 DOI: 10.1007/978-1-4614-5894-4_10]
45 **Wei Y**, Jiang Y, Zou F, Liu Y, Wang S, Xu N, Xu W, Cui C, Xing Y, Liu Y, Cao B, Liu C, Wu G, Ao H, Zhang X, Jiang J. Activation of PI3K/Akt pathway by CD133-p85 interaction promotes tumorigenic capacity of glioma stem cells. *Proc Natl Acad Sci USA* 2013; **110**: 6829-6834 [PMID: 23569237 DOI: 10.1073/pnas.1217002110]
46 **Wang YK**, Zhu YL, Qiu FM, Zhang T, Chen ZG, Zheng S, Huang J. Activation of Akt and MAPK pathways enhances the tumorigenicity of CD133+ primary colon cancer cells. *Carcinogenesis* 2010; **31**: 1376-1380 [PMID: 20530554 DOI: 10.1093/carcin/bgq120]
47 **Wang JY**, Chang CC, Chiang CC, Chen WM, Hung SC. Silibinin suppresses the maintenance of colorectal cancer stem-like cells by inhibiting PP2A/AKT/mTOR pathways. *J Cell Biochem* 2012; **113**: 1733-1743 [PMID: 22213051 DOI: 10.1002/jcb.24043]
48 **Corbo C**, Orrù S, Gemei M, Noto RD, Mirabelli P, Imperlini E, Ruoppolo M, Vecchio LD, Salvatore F. Protein cross-talk in CD133+ colon cancer cells indicates activation of the Wnt pathway and upregulation of SRp20 that is potentially involved in tumorigenicity. *Proteomics* 2012; **12**: 2045-2059 [PMID: 22623141 DOI: 10.1002/pmic.201100370]
49 **Ou J**, Deng J, Wei X, Xie G, Zhou R, Yu L, Liang H. Fibronectin extra domain A (EDA) sustains CD133(+)/CD44(+) subpopulation of colorectal cancer cells. *Stem Cell Res* 2013; **11**: 820-833 [PMID: 23811539 DOI: 10.1016/j.scr.2013.05.009]
50 **Mak AB**, Nixon AM, Kittanakom S, Stewart JM, Chen GI, Curak J, Gingras AC, Mazitschek R, Neel BG, Stagljar I, Moffat J. Regulation of CD133 by HDAC6 promotes β-catenin signaling to suppress cancer cell differentiation. *Cell Rep* 2012; **2**: 951-963 [PMID: 23084749 DOI: 10.1016/j.celrep.2012.09.016]
51 **Baba Y**, Nosho K, Shima K, Irahara N, Chan AT, Meyerhardt JA, Chung DC, Giovannucci EL, Fuchs CS, Ogino S. HIF1A overexpression is associated with poor prognosis in a cohort of 731 colorectal cancers. *Am J Pathol* 2010; **176**: 2292-2301 [PMID: 20363910 DOI: 10.2353/ajpath.2010.090972]
52 **Ohnishi S**, Maehara O, Nakagawa K, Kameya A, Otaki K, Fujita H, Higashi R, Takagi K, Asaka M, Sakamoto N, Kobayashi M, Takeda H. hypoxia-inducible factors activate CD133 promoter through ETS family transcription factors. *PLoS One* 2013; **8**: e66255 [PMID: 23840432 DOI: 10.1371/journal.pone.0066255]
53 **Mao Q**, Zhang Y, Fu X, Xue J, Guo W, Meng M, Zhou Z, Mo X, Lu Y. A tumor hypoxic niche protects human colon cancer stem cells from chemotherapy. *J Cancer Res Clin Oncol* 2013; **139**: 211-222 [PMID: 23052691 DOI: 10.1007/s00432-012-1310-3]
54 **Yi JM**, Tsai HC, Glöckner SC, Lin S, Ohm JE, Easwaran H, James CD, Costello JF, Riggins G, Eberhart CG, Laterra J, Vescovi AL, Ahuja N, Herman JG, Schuebel KE, Baylin SB. Abnormal DNA methylation of CD133 in colorectal and glioblastoma tumors. *Cancer Res* 2008; **68**: 8094-8103 [PMID: 18829568 DOI: 10.1158/0008-5472.CAN-07-6208]
55 **Screaton GR**, Bell MV, Jackson DG, Cornelis FB, Gerth U, Bell JI. Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. *Proc Natl Acad Sci USA* 1992; **89**: 12160-12164 [PMID: 1465456 DOI: 10.1073/pnas.89.24.12160]
56 **Sneath RJ**, Mangham DC. The normal structure and function of CD44 and its role in neoplasia. *Mol Pathol* 1998; **51**: 191-200 [PMID: 9893744 DOI: 10.1136/mp.51.4.191]
57 **Nagano O**, Saya H. Mechanism and biological significance of CD44 cleavage. *Cancer Sci* 2004; **95**: 930-935 [PMID: 15596040 DOI: 10.1111/j.1349-7006.2004.tb03179.x]
58 **Du L**, Wang H, He L, Zhang J, Ni B, Wang X, Jin H, Cahuzac N, Mehrpour M, Lu Y, Chen Q. CD44 is of functional importance for colorectal cancer stem cells. *Clin Cancer Res* 2008; **14**: 6751-6760 [PMID: 18980968 DOI: 10.1158/1078-0432.CCR-08-1034]
59 **Fan CW**, Chen T, Shang YN, Gu YZ, Zhang SL, Lu R, OuYang SR, Zhou X, Li Y, Meng WT, Hu JK, Lu Y, Sun XF, Bu H, Zhou ZG, Mo XM. Cancer-initiating cells derived from human rectal adenocarcinoma tissues carry mesenchymal phenotypes and resist drug therapies. *Cell Death Dis* 2013; **4**: e828 [PMID: 24091671 DOI: 10.1038/cddis.2013.337]
60 **Zeilstra J**, Joosten SP, Dokter M, Verwiel E, Spaargaren M,

Pals ST. Deletion of the WNT target and cancer stem cell marker CD44 in Apc(Min/+) mice attenuates intestinal tumorigenesis. *Cancer Res* 2008; **68**: 3655-3661 [PMID: 18483247 DOI: 10.1158/0008-5472.CAN-07-2940]

61 **Harada N**, Mizoi T, Kinouchi M, Hoshi K, Ishii S, Shiiba K, Sasaki I, Matsuno S. Introduction of antisense CD44S CDNA down-regulates expression of overall CD44 isoforms and inhibits tumor growth and metastasis in highly metastatic colon carcinoma cells. *Int J Cancer* 2001; **91**: 67-75 [PMID: 11149422 DOI: 10.1002/1097-0215(20010101)91: 1<67::AID-IJC1011>3.0.CO;2-D]

62 **Dallas MR**, Liu G, Chen WC, Thomas SN, Wirtz D, Huso DL, Konstantopoulos K. Divergent roles of CD44 and carcinoembryonic antigen in colon cancer metastasis. *FASEB J* 2012; **26**: 2648-2656 [PMID: 22415308 DOI: 10.1096/fj.12-203786]

63 **Ropponen KM**, Eskelinen MJ, Lipponen PK, Alhava E, Kosma VM. Expression of CD44 and variant proteins in human colorectal cancer and its relevance for prognosis. *Scand J Gastroenterol* 1998; **33**: 301-309 [PMID: 9548625 DOI: 10.1080/00365529850170900]

64 **Ylagan LR**, Scholes J, Demopoulos R. Cd44: a marker of squamous differentiation in adenosquamous neoplasms. *Arch Pathol Lab Med* 2000; **124**: 212-215 [PMID: 10656728]

65 **Fernández JC**, Vizoso FJ, Corte MD, Gava RR, Corte MG, Suárez JP, García-Muñíz JL, García-Morán M. CD44s expression in resectable colorectal carcinomas and surrounding mucosa. *Cancer Invest* 2004; **22**: 878-885 [PMID: 15641486 DOI: 10.1081/CNV-200039658]

66 **Huh JW**, Kim HR, Kim YJ, Lee JH, Park YS, Cho SH, Joo JK. Expression of standard CD44 in human colorectal carcinoma: association with prognosis. *Pathol Int* 2009; **59**: 241-246 [PMID: 19351367 DOI: 10.1111/j.1440-1827.2009.02357.x]

67 **Lugli A**, Iezzi G, Hostettler I, Muraro MG, Mele V, Tornillo L, Carafa V, Spagnoli G, Terracciano L, Zlobec I. Prognostic impact of the expression of putative cancer stem cell markers CD133, CD166, CD44s, EpCAM, and ALDH1 in colorectal cancer. *Br J Cancer* 2010; **103**: 382-390 [PMID: 20606680 DOI: 10.1038/sj.bjc.6605762]

68 **Herlyn M**, Steplewski Z, Herlyn D, Koprowski H. Colorectal carcinoma-specific antigen: detection by means of monoclonal antibodies. *Proc Natl Acad Sci USA* 1979; **76**: 1438-1442 [PMID: 286328 DOI: 10.1073/pnas.76.3.1438]

69 **Went P**, Vasei M, Bubendorf L, Terracciano L, Tornillo L, Riede U, Kononen J, Simon R, Sauter G, Baeuerle PA. Frequent high-level expression of the immunotherapeutic target Ep-CAM in colon, stomach, prostate and lung cancers. *Br J Cancer* 2006; **94**: 128-135 [PMID: 16404366 DOI: 10.1038/sj.bjc.6602924]

70 **Baeuerle PA**, Gires O. EpCAM (CD326) finding its role in cancer. *Br J Cancer* 2007; **96**: 417-423 [PMID: 17211480 DOI: 10.1038/sj.bjc.6603494]

71 **Trzpis M**, McLaughlin PM, de Leij LM, Harmsen MC. Epithelial cell adhesion molecule: more than a carcinoma marker and adhesion molecule. *Am J Pathol* 2007; **171**: 386-395 [PMID: 17600130 DOI: 10.2353/ajpath.2007.070152]

72 **Denzel S**, Maetzel D, Mack B, Eggert C, Bärr G, Gires O. Initial activation of EpCAM cleavage via cell-to-cell contact. *BMC Cancer* 2009; **9**: 402 [PMID: 19925656 DOI: 10.1186/1471-2407-9-402]

73 **Balzar M**, Prins FA, Bakker HA, Fleuren GJ, Warnaar SO, Litvinov SV. The structural analysis of adhesions mediated by Ep-CAM. *Exp Cell Res* 1999; **246**: 108-121 [PMID: 9882520 DOI: 10.1006/excr.1998.4263]

74 **Ralhan R**, He HC, So AK, Tripathi SC, Kumar M, Hasan MR, Kaur J, Kashat L, MacMillan C, Chauhan SS, Freeman JL, Walfish PG. Nuclear and cytoplasmic accumulation of Ep-ICD is frequently detected in human epithelial cancers. *PLoS One* 2010; **5**: e14130 [PMID: 21152431 DOI: 10.1371/journal.pone.0014130]

75 **Dylla SJ**, Beviglia L, Park IK, Chartier C, Raval J, Ngan L, Pickell K, Aguilar J, Lazetic S, Smith-Berdan S, Clarke MF, Hoey T, Lewicki J, Gurney AL. Colorectal cancer stem cells are enriched in xenogeneic tumors following chemotherapy. *PLoS One* 2008; **3**: e2428 [PMID: 18560594 DOI: 10.1371/journal.pone.0002428]

76 **Kristiansen G**, Sammar M, Altevogt P. Tumour biological aspects of CD24, a mucin-like adhesion molecule. *J Mol Histol* 2004; **35**: 255-262 [PMID: 15339045 DOI: 10.1023/B:HIJO.0000032357.16261.c5]

77 **Nestl A**, Von Stein OD, Zatloukal K, Thies WG, Herrlich P, Hofmann M, Sleeman JP. Gene expression patterns associated with the metastatic phenotype in rodent and human tumors. *Cancer Res* 2001; **61**: 1569-1577 [PMID: 11245467]

78 **Weichert W**, Denkert C, Burkhardt M, Gansukh T, Bellach J, Altevogt P, Dietel M, Kristiansen G. Cytoplasmic CD24 expression in colorectal cancer independently correlates with shortened patient survival. *Clin Cancer Res* 2005; **11**: 6574-6581 [PMID: 16166435 DOI: 10.1158/1078-0432.CCR-05-0606]

79 **Choi D**, Lee HW, Hur KY, Kim JJ, Park GS, Jang SH, Song YS, Jang KS, Paik SS. Cancer stem cell markers CD133 and CD24 correlate with invasiveness and differentiation in colorectal adenocarcinoma. *World J Gastroenterol* 2009; **15**: 2258-2264 [PMID: 19437567 DOI: 10.3748/wjg.15.2258]

80 **Yeung TM**, Gandhi SC, Wilding JL, Muschel R, Bodmer WF. Cancer stem cells from colorectal cancer-derived cell lines. *Proc Natl Acad Sci USA* 2010; **107**: 3722-3727 [PMID: 20133591 DOI: 10.1073/pnas.0915135107]

81 **Muppala S**, Mudduluru G, Leupold JH, Buergy D, Sleeman JP, Allgayer H. CD24 induces expression of the oncomir miR-21 via Src, and CD24 and Src are both post-transcriptionally downregulated by the tumor suppressor miR-34a. *PLoS One* 2013; **8**: e59563 [PMID: 23533633 DOI: 10.1371/journal.pone.0059563]

82 **Brizzi MF**, Tarone G, Defilippi P. Extracellular matrix, integrins, and growth factors as tailors of the stem cell niche. *Curr Opin Cell Biol* 2012; **24**: 645-651 [PMID: 22898530 DOI: 10.1016/j.ceb.2012.07.001]

83 **Hynes RO**. Integrins: bidirectional, allosteric signaling machines. *Cell* 2002; **110**: 673-687 [PMID: 12297042 DOI: 10.1016/S0092-8674(02)00971-6]

84 **Pignatelli M**, Liu D, Nasim MM, Stamp GW, Hirano S, Takeichi M. Morphoregulatory activities of E-cadherin and beta-1 integrins in colorectal tumour cells. *Br J Cancer* 1992; **66**: 629-634 [PMID: 1384639 DOI: 10.1038/bjc.1992.328]

85 **Okazaki K**, Nakayama Y, Shibao K, Hirata K, Nagata N, Itoh H. Enhancement of metastatic activity of colon cancer as influenced by expression of cell surface antigens. *J Surg Res* 1998; **78**: 78-84 [PMID: 9733622 DOI: 10.1006/jsre.1998.5298]

86 **Fan LF**, Dong WG, Jiang CQ, Xia D, Liao F, Yu QF. Expression of putative stem cell genes Musashi-1 and beta1-integrin in human colorectal adenomas and adenocarcinomas. *Int J Colorectal Dis* 2010; **25**: 17-23 [PMID: 19714342 DOI: 10.1007/s00384-009-0791-2]

87 **Langan RC**, Mullinax JE, Ray S, Raiji MT, Schaub N, Xin HW, Koizumi T, Steinberg SM, Anderson A, Wiegand G, Butcher D, Anver M, Bilchik AJ, Stojadinovic A, Rudloff U, Avital I. A Pilot Study Assessing the Potential Role of non-CD133 Colorectal Cancer Stem Cells as Biomarkers. *J Cancer* 2012; **3**: 231-240 [PMID: 22670157 DOI: 10.7150/jca.4542]

88 **Hongo K**, Tanaka J, Tsuno NH, Kawai K, Nishikawa T, Shuno Y, Sasaki K, Kaneko M, Hiyoshi M, Sunami E, Kitayama J, Takahashi K, Nagawa H. CD133(-) cells, derived from a single human colon cancer cell line, are more resistant to 5-fluorouracil (FU) than CD133(+) cells, dependent on the β1-integrin signaling. *J Surg Res* 2012; **175**: 278-288 [PMID: 21601882 DOI: 10.1016/j.jss.2011.03.076]

89 **Van der Flier LG**, Sabates-Bellver J, Oving I, Haegebarth A, De Palo M, Anti M, Van Gijn ME, Suijkerbuijk S, Van

de Wetering M, Marra G, Clevers H. The Intestinal Wnt/TCF Signature. *Gastroenterology* 2007; **132**: 628-632 [PMID: 17320548 DOI: 10.1053/j.gastro.2006.08.039]

90 **de Lau WB**, Snel B, Clevers HC. The R-spondin protein family. *Genome Biol* 2012; **13**: 242 [PMID: 22439850 DOI: 10.1186/gb-2012-13-3-242]

91 **van de Wetering M**, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der Horn K, Batlle E, Coudreuse D, Haramis AP, Tjon-Pon-Fong M, Moerer P, van den Born M, Soete G, Pals S, Eilers M, Medema R, Clevers H. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* 2002; **111**: 241-250 [PMID: 12408868 DOI: 10.1016/S0092-8674(02)01014-0]

92 **Wu XS**, Xi HQ, Chen L. Lgr5 is a potential marker of colorectal carcinoma stem cells that correlates with patient survival. *World J Surg Oncol* 2012; **10**: 244 [PMID: 23153436 DOI: 10.1186/1477-7819-10-244]

93 **Haegebarth A**, Clevers H. Wnt signaling, lgr5, and stem cells in the intestine and skin. *Am J Pathol* 2009; **174**: 715-721 [PMID: 19197002 DOI: 10.2353/ajpath.2009.080758]

94 **Takahashi H**, Ishii H, Nishida N, Takemasa I, Mizushima T, Ikeda M, Yokobori T, Mimori K, Yamamoto H, Sekimoto M, Doki Y, Mori M. Significance of Lgr5(+ve) cancer stem cells in the colon and rectum. *Ann Surg Oncol* 2011; **18**: 1166-1174 [PMID: 21125339 DOI: 10.1245/s10434-010-1373-9]

95 **Batlle E**, Henderson JT, Beghtel H, van den Born MM, Sancho E, Huls G, Meeldijk J, Robertson J, van de Wetering M, Pawson T, Clevers H. Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. *Cell* 2002; **111**: 251-263 [PMID: 12408869 DOI: 10.1016/S0092-8674(02)01015-2]

96 **Lehmann JM**, Riethmüller G, Johnson JP. MUC18, a marker of tumor progression in human melanoma, shows sequence similarity to the neural cell adhesion molecules of the immunoglobulin superfamily. *Proc Natl Acad Sci USA* 1989; **86**: 9891-9895 [PMID: 2602381 DOI: 10.1073/pnas.86.24.9891]

97 **Aruffo A**, Bowen MA, Patel DD, Haynes BF, Starling GC, Gebe JA, Bajorath J. CD6-ligand interactions: a paradigm for SRCR domain function? *Immunol Today* 1997; **18**: 498-504 [PMID: 9357143 DOI: 10.1016/S0167-5699(97)01130-4]

98 **Weidle UH**, Eggle D, Klostermann S, Swart GW. ALCAM/CD166: cancer-related issues. *Cancer Genomics Proteomics* 2010; **7**: 231-243 [PMID: 20952758]

99 **Levin TG**, Powell AE, Davies PS, Silk AD, Dismuke AD, Anderson EC, Swain JR, Wong MH. Characterization of the intestinal cancer stem cell marker CD166 in the human and mouse gastrointestinal tract. *Gastroenterology* 2010; **139**: 2072-2082.e5 [PMID: 20826154 DOI: 10.1053/j.gastro.2010.08.053]

100 **Weichert W**, Knösel T, Bellach J, Dietel M, Kristiansen G. ALCAM/CD166 is overexpressed in colorectal carcinoma and correlates with shortened patient survival. *J Clin Pathol* 2004; **57**: 1160-1164 [PMID: 15509676 DOI: 10.1136/jcp.2004.016238]

101 **Horst D**, Kriegl L, Engel J, Kirchner T, Jung A. Prognostic significance of the cancer stem cell markers CD133, CD44, and CD166 in colorectal cancer. *Cancer Invest* 2009; **27**: 844-850 [PMID: 19626493 DOI: 10.1080/07357900902744502]

102 **Tachezy M**, Zander H, Gebauer F, Marx A, Kaifi JT, Izbicki JR, Bockhorn M. Activated leukocyte cell adhesion molecule (CD166)--its prognostic power for colorectal cancer patients. *J Surg Res* 2012; **177**: e15-e20 [PMID: 22482754 DOI: 10.1016/j.jss.2012.02.013]

103 **Frank NY**, Schatton T, Frank MH. The therapeutic promise of the cancer stem cell concept. *J Clin Invest* 2010; **120**: 41-50 [PMID: 20051635 DOI: 10.1172/JCI41004]

104 **Todaro M**, Alea MP, Di Stefano AB, Cammareri P, Vermeulen L, Iovino F, Tripodo C, Russo A, Gulotta G, Medema JP, Stassi G. Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. *Cell Stem Cell* 2007; **1**: 389-402 [PMID: 18371377 DOI: 10.1016/j.stem.2007.08.001]

105 **Dallas NA**, Xia L, Fan F, Gray MJ, Gaur P, van Buren G, Samuel S, Kim MP, Lim SJ, Ellis LM. Chemoresistant colorectal cancer cells, the cancer stem cell phenotype, and increased sensitivity to insulin-like growth factor-I receptor inhibition. *Cancer Res* 2009; **69**: 1951-1957 [PMID: 19244128 DOI: 10.1158/0008-5472.CAN-08-2023]

106 **Bach P**, Abel T, Hoffmann C, Gal Z, Braun G, Voelker I, Ball CR, Johnston IC, Lauer UM, Herold-Mende C, Mühlebach MD, Glimm H, Buchholz CJ. Specific elimination of CD133+ tumor cells with targeted oncolytic measles virus. *Cancer Res* 2013; **73**: 865-874 [PMID: 23293278 DOI: 10.1158/0008-5472.CAN-12-2221]

107 **Bostad M**, Berg K, Høgset A, Skarpen E, Stenmark H, Selbo PK. Photochemical internalization (PCI) of immunotoxins targeting CD133 is specific and highly potent at femtomolar levels in cells with cancer stem cell properties. *J Control Release* 2013; **168**: 317-326 [PMID: 23567040 DOI: 10.1016/j.jconrel.2013.03.023]

108 **Chen H**, Luo Z, Sun W, Zhang C, Sun H, Zhao N, Ding J, Wu M, Li Z, Wang H. Low glucose promotes CD133mAb-elicited cell death via inhibition of autophagy in hepatocarcinoma cells. *Cancer Lett* 2013; **336**: 204-212 [PMID: 23652197 DOI: 10.1016/j.canlet.2013.04.031]

109 **Swaminathan SK**, Roger E, Toti U, Niu L, Ohlfest JR, Panyam J. CD133-targeted paclitaxel delivery inhibits local tumor recurrence in a mouse model of breast cancer. *J Control Release* 2013; **171**: 280-287 [PMID: 23871962 DOI: 10.1016/j.jconrel.2013.07.014]

110 **Riethmüller G**, Schneider-Gädicke E, Schlimok G, Schmiegel W, Raab R, Höffken K, Gruber R, Pichlmaier H, Hirche H, Pichlmayr R. Randomised trial of monoclonal antibody for adjuvant therapy of resected Dukes' C colorectal carcinoma. German Cancer Aid 17-1A Study Group. *Lancet* 1994; **343**: 1177-1183 [PMID: 7909866 DOI: 10.1016/S0140-6736(94)92398-1]

111 **Punt CJ**, Nagy A, Douillard JY, Figer A, Skovsgaard T, Monson J, Barone C, Fountzilas G, Riess H, Moylan E, Jones D, Dethling J, Colman J, Coward L, MacGregor S. Edrecolomab alone or in combination with fluorouracil and folinic acid in the adjuvant treatment of stage III colon cancer: a randomised study. *Lancet* 2002; **360**: 671-677 [PMID: 12241873 DOI: 10.1016/S0140-6736(02)09836-7]

112 **Fields AL**, Keller A, Schwartzberg L, Bernard S, Kardinal C, Cohen A, Schulz J, Eisenberg P, Forster J, Wissel P. Adjuvant therapy with the monoclonal antibody Edrecolomab plus fluorouracil-based therapy does not improve overall survival of patients with stage III colon cancer. *J Clin Oncol* 2009; **27**: 1941-1947 [PMID: 19273708 DOI: 10.1200/JCO.2008.18.5710]

113 **Waldron NN**, Barsky SH, Dougherty PR, Vallera DA. A bispecific EpCAM/CD133-targeted toxin is effective against carcinoma. *Target Oncol* 2013; Epub ahead of print [PMID: 23900680]

114 **Misra S**, Hascall VC, De Giovanni C, Markwald RR, Ghatak S. Delivery of CD44 shRNA/nanoparticles within cancer cells: perturbation of hyaluronan/CD44v6 interactions and reduction in adenoma growth in Apc Min/+ MICE. *J Biol Chem* 2009; **284**: 12432-12446 [PMID: 19246453 DOI: 10.1074/jbc.M806772200]

115 **Nautiyal J**, Kanwar SS, Yu Y, Majumdar AP. Combination of dasatinib and curcumin eliminates chemo-resistant colon cancer cells. *J Mol Signal* 2011; **6**: 7 [PMID: 21774804 DOI: 10.1186/1750-2187-6-7]

116 **Kanwar SS**, Yu Y, Nautiyal J, Patel BB, Padhye S, Sarkar FH, Majumdar AP. Difluorinated-curcumin (CDF): a novel curcumin analog is a potent inhibitor of colon cancer stem-like cells. *Pharm Res* 2011; **28**: 827-838 [PMID: 21161336 DOI: 10.1007/s11095-010-0336-y]

117 **Yu M**, Jambhrunkar S, Thorn P, Chen J, Gu W, Yu C. Hy-

aluronic acid modified mesoporous silica nanoparticles for targeted drug delivery to CD44-overexpressing cancer cells. *Nanoscale* 2013; **5**: 178-183 [PMID: 23076766 DOI: 10.1039/c2nr32145a]

118 **Sagiv E**, Starr A, Rozovski U, Khosravi R, Altevogt P, Wang T, Arber N. Targeting CD24 for treatment of colorectal and pancreatic cancer by monoclonal antibodies or small interfering RNA. *Cancer Res* 2008; **68**: 2803-2812 [PMID: 18413748 DOI: 10.1158/0008-5472.CAN-07-6463]

119 **Park JJ**, Seo SM, Kim EJ, Lee YJ, Ko YG, Ha J, Lee M. Berberine inhibits human colon cancer cell migration via AMP-activated protein kinase-mediated downregulation of integrin β1 signaling. *Biochem Biophys Res Commun* 2012; **426**: 461-467 [PMID: 22943849 DOI: 10.1016/j.bbrc.2012.08.091]

120 **Behrens J**, von Kries JP, Kühl M, Bruhn L, Wedlich D, Grosschedl R, Birchmeier W. Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* 1996; **382**: 638-642 [PMID: 8757136 DOI: 10.1038/382638a0]

121 **Chen B**, Dodge ME, Tang W, Lu J, Ma Z, Fan CW, Wei S, Hao W, Kilgore J, Williams NS, Roth MG, Amatruda JF, Chen C, Lum L. Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. *Nat Chem Biol* 2009; **5**: 100-107 [PMID: 19125156 DOI: 10.1038/nchembio.137]

122 **Huang SM**, Mishina YM, Liu S, Cheung A, Stegmeier F, Michaud GA, Charlat O, Wiellette E, Zhang Y, Wiessner S, Hild M, Shi X, Wilson CJ, Mickanin C, Myer V, Fazal A, Tomlinson R, Serluca F, Shao W, Cheng H, Shultz M, Rau C, Schirle M, Schlegl J, Ghidelli S, Fawell S, Lu C, Curtis D, Kirschner MW, Lengauer C, Finan PM, Tallarico JA, Bouwmeester T, Porter JA, Bauer A, Cong F. Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. *Nature* 2009; **461**: 614-620 [PMID: 19759537 DOI: 10.1038/nature08356]

123 **Thorne CA**, Hanson AJ, Schneider J, Tahinci E, Orton D, Cselenyi CS, Jernigan KK, Meyers KC, Hang BI, Waterson AG, Kim K, Melancon B, Ghidu VP, Sulikowski GA, LaFleur B, Salic A, Lee LA, Miller DM, Lee E. Small-molecule inhibition of Wnt signaling through activation of casein kinase 1α. *Nat Chem Biol* 2010; **6**: 829-836 [PMID: 20890287 DOI: 10.1038/nchembio.453]

124 **Gonsalves FC**, Klein K, Carson BB, Katz S, Ekas LA, Evans S, Nagourney R, Cardozo T, Brown AM, DasGupta R. An RNAi-based chemical genetic screen identifies three small-molecule inhibitors of the Wnt/wingless signaling pathway. *Proc Natl Acad Sci USA* 2011; **108**: 5954-5963 [PMID: 21393571 DOI: 10.1073/pnas.1017496108]

125 **Emami KH**, Nguyen C, Ma H, Kim DH, Jeong KW, Eguchi M, Moon RT, Teo JL, Kim HY, Moon SH, Ha JR, Kahn M. A small molecule inhibitor of beta-catenin/CREB-binding protein transcription [corrected]. *Proc Natl Acad Sci USA* 2004; **101**: 12682-12687 [PMID: 15314234 DOI: 10.1073/pnas.0404875101]

126 **Takahashi-Yanaga F**, Kahn M. Targeting Wnt signaling: can we safely eradicate cancer stem cells? *Clin Cancer Res* 2010; **16**: 3153-3162 [PMID: 20530697 DOI: 10.1158/1078-0432.CCR-09-2943]

127 **He B**, You L, Uematsu K, Xu Z, Lee AY, Matsangou M, McCormick F, Jablons DM. A monoclonal antibody against Wnt-1 induces apoptosis in human cancer cells. *Neoplasia* 2004; **6**: 7-14 [PMID: 15068666]

128 **Ueno K**, Hazama S, Mitomori S, Nishioka M, Suehiro Y, Hirata H, Oka M, Imai K, Dahiya R, Hinoda Y. Down-regulation of frizzled-7 expression decreases survival, invasion and metastatic capabilities of colon cancer cells. *Br J Cancer* 2009; **101**: 1374-1381 [PMID: 19773752 DOI: 10.1038/sj.bjc.6605307]

129 **Anastas JN**, Moon RT. WNT signalling pathways as therapeutic targets in cancer. *Nat Rev Cancer* 2013; **13**: 11-26 [PMID: 23258168 DOI: 10.1038/nrc3419]

130 **Hoey T**, Yen WC, Axelrod F, Basi J, Donigian L, Dylla S, Fitch-Bruhns M, Lazetic S, Park IK, Sato A, Satyal S, Wang X, Clarke MF, Lewicki J, Gurney A. DLL4 blockade inhibits tumor growth and reduces tumor-initiating cell frequency. *Cell Stem Cell* 2009; **5**: 168-177 [PMID: 19664991 DOI: 10.1016/j.stem.2009.05.019]

131 **Fischer M**, Yen WC, Kapoun AM, Wang M, O'Young G, Lewicki J, Gurney A, Hoey T. Anti-DLL4 inhibits growth and reduces tumor-initiating cell frequency in colorectal tumors with oncogenic KRAS mutations. *Cancer Res* 2011; **71**: 1520-1525 [PMID: 21193546 DOI: 10.1158/0008-5472.CAN-10-2817]

P- Reviewers: Bueno V, Dittmar T, Li YC, Zhang Q
S- Editor:Cui XM **L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.943

World J Gastroenterol 2014 January 28; 20(4): 943-956
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Genetic and epigenetic biomarkers for diagnosis, prognosis and treatment of colorectal cancer

Fabio Coppedè, Angela Lopomo, Roberto Spisni, Lucia Migliore

Fabio Coppedè, Angela Lopomo, Lucia Migliore, Department of Translational Research and New Technologies in Medicine and Surgery, Division of Medical Genetics, University of Pisa, 56126 Pisa, Italy
Fabio Coppedè, Lucia Migliore, Istituto Toscano Tumori (ITT), 50139 Florence, Italy
Roberto Spisni, Department of Surgery, Medical, Molecular, and Critical Area Pathology, University of Pisa, 56126 Pisa, Italy
Author contributions: Coppedè F wrote the introduction and the sections of epigenetic biomarkers and future perspectives; Lopomo A wrote the section of genetic biomarkers; Spisni R and Migliore L critically revised the entire manuscript.
Supported by T Istituto Toscano Tumori (ITT) No. Prot. AOOGRT/325424/Q.80.110 16/12/2009, "Correlation among epigenetic, environmental and genetic factors in colorectal carcinoma"
Correspondence to: Lucia Migliore, Professor, Department of Translational Research and New Technologies in Medicine and Surgery, Division of Medical Genetics, University of Pisa, Via S. Giuseppe 22, 56126 Pisa, Italy. lucia.migliore@med.unipi.it
Telephone: +39-50-2211029 Fax: +39-50-2211034
Received: October 2, 2013 Revised: November 21, 2013
Accepted: December 5, 2013
Published online: January 28, 2014

Abstract

Colorectal cancer (CRC) is one of the most common cancer worldwide and results from the accumulation of mutations and epimutations in colonic mucosa cells ultimately leading to cell proliferation and metastasis. Unfortunately, CRC prognosis is still poor and the search of novel diagnostic and prognostic biomarkers is highly desired to prevent CRC-related deaths. The present article aims to summarize the most recent findings concerning the use of either genetic or epigenetic (mainly related to DNA methylation) biomarkers for CRC diagnosis, prognosis, and response to treatment. Recent large-scale DNA methylation studies suggest that CRC can be divided into several subtypes according to the frequency of DNA methylation and those of mutations in key CRC genes, and that this is reflected by different prognostic outcomes. Increasing evidence suggests that the analysis of DNA methylation in blood or fecal specimens could represent a valuable non-invasive diagnostic tool for CRC. Moreover, a broad spectrum of studies indicates that the inter-individual response to chemotherapeutic treatments depends on both epigenetic modifications and genetic mutations occurring in colorectal cancer cells, thereby opening the way for a personalized medicine. Overall, combining genetic and epigenetic data might represent the most promising tool for a proper diagnostic, prognostic and therapeutic approach.

Key words: Colorectal cancer; Genetic biomarkers; Epigenetic biomarkers; DNA methylation; Diagnostic biomarkers; *APC*; *MGMT*; *KRAS*

Core tip: We summarize the most recent findings concerning genetic and epigenetic biomarkers of colorectal cancer. The article aims to provide an overview of the currently available diagnostic and prognostic biomarkers of the disease. Attention is also paid to the possible application of those biomarkers for the choice of the most proper therapy.

Coppedè F, Lopomo A, Spisni R, Migliore L. Genetic and epigenetic biomarkers for diagnosis, prognosis and treatment of colorectal cancer. *World J Gastroenterol* 2014; 20(4): 943-956 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/943.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.943

INTRODUCTION

It is now clear that cancer is a multi-step process resulting

from the accumulation of both genetic and epigenetic alterations of the genome[1]. Gene mutations and epigenetic modifications have been initially viewed as two separate mechanisms participating in carcinogenesis. However recent evidence points to a crosstalk between these two mechanisms in cancer formation, suggesting that gene mutations have the potential of disrupting several epigenetic patterns and that epigenetic modifications can drive genome instability and mutagenesis[2,3]. For example, the whole exome sequencing of thousands of human cancers revealed unexpected mutations in genes involved in epigenetic mechanisms, and those mutations have the potential to disrupt DNA methylation patterns, histone modifications, and nucleosome positioning[3]. Similarly, epigenetic inactivation of DNA repair genes, such as h*MLH1*, h*MSH2*, *MGMT* and *BRCA1*, is often associated with genome instability and increased frequency of point mutations of cancer-related genes[2].

Colorectal cancer (CRC) is one of the most frequent cancers in humans, with over one-million new cases diagnosed worldwide every year[4]. The disease occurs sporadically in most of the cases (75%-80%) as a result of the accumulation of both mutations and epigenetic modifications of several genes[5], and large-scale DNA methylation studies suggest that CRC can be divided into at least three-four subtypes according to the frequency of DNA methylation and those of mutations in key CRC genes[6,7]. The sequential process of gene mutations and epigenetic alterations is believed to drive the progression toward malignant adeno-carcinomas because those events affect signalling pathways that regulate hallmark behaviours of cancer. Gene mutations create a clonal growth advantage that leads to the outgrowth of progressively more malignant cells, which ultimately manifests itself as invasive adeno-carcinoma. The 5-year survival rates are approximately 90% for early CRC patients but decrease to less than 10% in patients with distant metastases, by this the need to identify biomarkers to improve the prediction of clinical outcomes in CRC[8]. Further progress is very much desirable in non-invasive diagnostic methods to enable early diagnosis, pre- and postoperative staging, and to assist in selecting the most suitable neo-adjuvant and adjuvant therapeutic methods and post-treatment. Novel biomarkers which are absent in healthy persons and present in CRC are still being investigated, especially those that can be detected at early development stage of the disease and used in screening tests. Unfortunately, no molecule that would meet all of the foregoing criteria has been identified so far. Carcinoembryonic antigen still remains the only tumour marker of recognised efficacy in monitoring patients during and after CRC therapy[9].

There is an increasing interest to identify mutations in key genes of tumourigenesis, such as *APC*, *CTNNB1*, *BRAF* and *KRAS* because they are involved in the Wnt and the Ras-Raf-MEK-MAPK signalling cascades (MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase) and therefore play a substantial role in the adenoma-carcinoma and in the serrated adenoma pathways. There are also attempts to "personalise" chemotherapy based on presence or absence of specific genetic biomarkers. For example, therapy with anti-EGFR (epidermal growth factor receptor) antibodies is desirable in patients with advanced CRC and absence of *KRAS* or *BRAF* mutations, and defining tumours phenotype - microsatellite instability (MSI) or microsatellite stability (MSS) and testing for the presence or absence of 18q chromosome deletion is very much desirable in standard 5-fluorouracil (5-FU)-based therapy[9,10].

DNA methylation represents one of the most studied epigenetic marks in CRC[11], since methylation of CpG islands in the promoter region of a gene might induce chromatin conformational modifications and inhibit the access of the transcriptional machinery, thus altering gene expression levels. Promoter hypermethylation is commonly associated with gene silencing as well as promoter demethylation with gene expression. The ever-growing number of genes that show epigenetic alterations in cancer emphasizes the crucial role of these epigenetic alterations, and particularly of DNA methylation, for future diagnosis, prognosis and prediction of response to therapies[12]. Lao *et al*[11] (2011) reviewed the genes that seem to be more commonly methylated in the multi-step process leading from normal colonic epithelium to adenocarcinoma, observing that some of them are frequently methylated in the passage from a normal colon epithelium to an aberrant crypt focus, whilst others are methylated in the passage from an aberrant crypt focus to polyp/adenoma, or could have a role in CRC progression and metastasis. Concerning CRC diagnosis, there is increasing interest in searching for aberrantly methylated genes in plasma DNA and in the DNA obtained from faecal material, as non-invasive diagnostic tools[13,14]. Methylation of certain genes, such as for example those involved in the extracellular matrix (ECM) remodelling pathway, were associated with worse survival in CRC, suggesting that epigenetic biomarkers could gain prognostic value[15]. There is also active research focusing on epigenetic signatures in CRC for their possible interaction with chemotherapeutic agents[16].

Given the enormous potential of both gene mutations and DNA methylation biomarkers in CRC diagnosis, staging, prognosis and response to treatment, active research is currently ongoing to develop rapid, cost effective and reproducible tools for the detection of those marks[12]. Aim of this article is to review currently available genetic and DNA methylation biomarkers for CRC diagnosis, staging, prognosis and treatment.

GENETIC BIOMARKERS IN CRC

Genetic and cytogenetic biomarkers

In 1990, Fearon and Vogelstein proposed a model for colorectal cancer tumourigenesis, which defines the genetic alterations involved in transformation from normal intestinal mucosa to colorectal carcinoma. This aberrant transformation is a multi-step process that includes genet-

ic alterations such as mutation of the *APC* (adenomatous polyposis coli gene), located on chromosome 5q, which is thought to occur early on during the development of adenomatous polyps, the activation of *KRAS* (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog gene), an oncogene located on chromosome 12p12, during the adenomatous stage and loss of chromosomal regions 17p and 18q that contain tumoural suppressor genes as tumour protein p53 (*TP53*) and *DCC* (deleted in colorectal carcinoma), in the transition to carcinoma *in situ*[17]. A lot of studies, by different approaches, identified these common alterations described by Fearon and Vogelstein and, in addition, others changes such as gain of chromosomes 7, 8q, 13q and 20q, together with loss of the 1p, 4,8p and 22q chromosomal regions were also identified[18-20]. Some studies have suggested that the loss of heterozygosity (LOH) of 17p and 18q could be associated with more advanced stages of the disease; the loss of 17p and 18q are believed to play an important role in the pathogenesis of CRC since these two chromosomes carry genes relevant to the malignant transformation of the gut epithelium and also probably play an important role in the metastatic process. In this regard, recent findings showed that breakpoints in the 17p11.2 chromosomal region were preferentially found in primary colonic tumours in CRC patients with liver metastases[21,22]. The deletion of the long arm of chromosome 18 (loss of 18q or LOH of 18q) is the most common cytogenetic abnormality in CRC and seems to be associated with poor prognosis as 18q contains several important tumour suppressor genes, such as *SMAD7*, *SMAD4*, and *SMAD2* that are transcriptional mediators in the TGF-β signalling pathway and *DCC*[23,24]. Mouse studies demonstrate that loss of *SMAD4* expression changes the role of TGF-β from growth suppressor to growth promoter, thus increasing the tumorigenic and metastatic potential of colorectal cancer cells[25]. Loss of SMAD activity occurs in 10% of the colorectal cancers and is associated with advanced-stage disease, the presence of lymph node metastases and shorter overall survival and it has been shown to be a significant independent prognostic factor for worse recurrence-free and overall survival, particularly in patients with stage Ⅲ disease. Patients with stage Ⅲ disease and intact *SMAD4* expression with microsatellite instability were found to have similar outcomes compared with patients with stage Ⅱ disease, whereas patients with stage Ⅱ disease and loss of *SMAD4* expression without microsatellite instability status had outcomes similar to patients with stage Ⅲ disease[26]. Retention of *SMAD4* expression has also been found to be a predictive marker for a threefold increase in benefit from 5-FU-based chemotherapy[27] while the loss of *SMAD4* seems to be a predictive marker for a poorer response to 5-FU[28]. So this chromosome instability (CIN) could have a prognostic value, as patients with CIN+ disease have a poorer prognosis[29].

Microsatellite Instability

Over the CIN, another form of genomic instability frequent in CRC is the microsatellite instability, observed at the nucleotide level, frequently resulting in deletions or insertions of a few nucleotides. Microsatellites are polymorphic tandem repeats of short nucleotide sequences distributed through the genome prone to frame shifts and base-pair substitutions during replication if DNA mismatch repair (MMR) genes are impaired. So MSI refers to a clonal change in the number of repeated DNA nucleotide units in microsatellites and appears in tumours with deficient mismatch repair due to the inactivation of the four MMR genes: *MSH2*, *MLH1*, *MSH6* and *PMS2* and while it is typically associated with hereditary non-polyposis colorectal cancer (HNPCC), most MSI-high tumours occur sporadically[30]. Sporadic MSI tumours tend to be more proximal, to occur in older females, to be poorly differentiated and mucinous, and to show marked lymphocytic infiltration[31,32]. Despite their resistance to alkylating agents and cisplatin, MSI-high tumours have better recurrence-free and overall survival. In patients with stage Ⅱ disease, MSI-high status was found to confer the same advantage in long-term outcomes as that conferred by stage T3 over T4[26]. MSI positive tumours are associated with a better prognosis in all stages of the disease. Patients with MSI tumours have a significant survival advantage compared with patients with non-MSI tumours[33] and are associated with resistance to 5-FU chemotherapy and shorter survival of patients after treatment with the drug[34,35]. MSI can be thus seen as one of the most promising positive prognostic markers for CRC patients and can be detected using a panel of five markers (*BAT25*, *BAT26*, *D2S123*, *D5S346*, and *D17S2720*, particularly analyzing this 5 loci, MSI-H is defined as instability at 2 loci or more, and MSI-L, as instability at 1 locus) and a recent study using this panel observed that the presence of MSI-H was significantly higher in carcinomas than in adenomas, confirming the prognostic value of MSI in CRC[36].

APC gene

Mutations in the *APC* gene are responsible for familial adenomatous polyposis (FAP) and the majority of sporadic CRC. The *APC* gene encodes a multifunctional protein with important roles in Wnt signaling pathway, intercellular adhesion, cytoskeleton stabilization, cell cycle regulation, and apoptosis. Mutations of *APC* may lead to unregulated transcription of oncogenes such as c-myc and cyclin D1, thereby promoting tumourigenesis. Mutations in Wnt/APC/CTNNB1(β-catenin) signalling pathway members have been found in many CRC and more than 90% of patients have alterations that affect it. In light of the critical role of the Wnt/APC/CTNNB1 signalling pathway in maintaining proper colorectal cell function, it is possible that genetic variants in this pathway might affect CRC progression. A meta-analysis provides a complete and systematic picture of the role of three *APC* polymorphisms (D1822V, E1317Q, I1307K) in the risk of colorectal neoplasia, particularly the I1307K variant was associated with a significantly increased risk

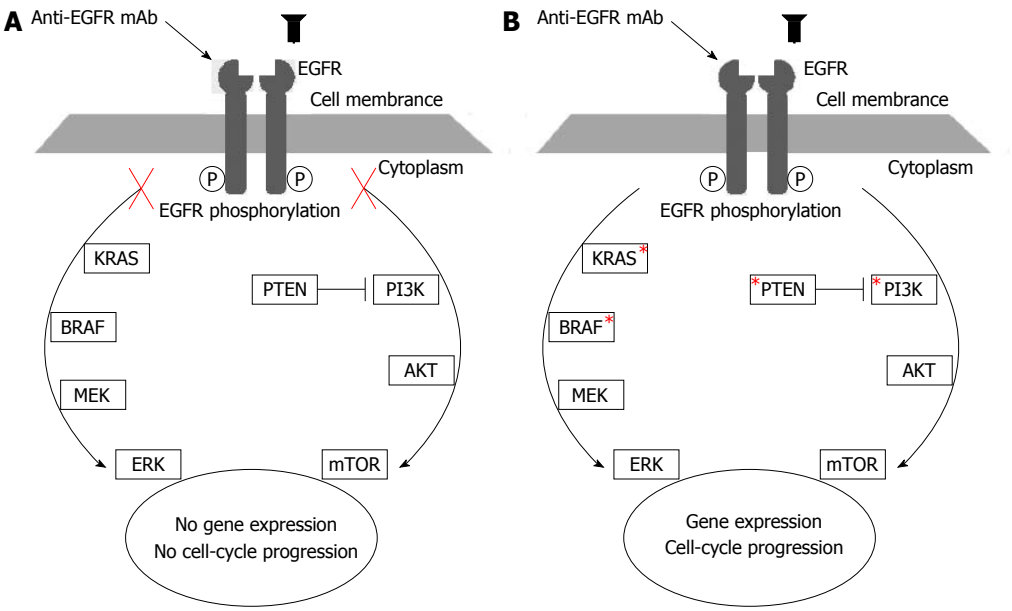


Figure 1 Representation of epidermal growth factor receptor pathway in response to therapy with anti-epidermal growth factor receptor inhibitors in wild-type and mutant patients. A: The binding of monoclonal antibodies (mAb) to EGFR normally causes the blockage (indicated with a red cross) of downstream RAS/RAF/MAPK and PI3K/AKT/mTOR signalling pathways and so the blockage of gene expression and cell cycle progression; B: Mutations in any gene of this pathway (indicated with a red star) cause a constitutive activation of the pathway leading to gene expression, upregulated proliferation, impaired differentiation and no response to monoclonal inhibitors. EGFR: Epidermal growth factor receptor; KRAS: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog gene; BRAF: V-raf murine sarcoma viral oncogenes homolog B1; MEK: MAPK/ERK kinase; ERK: Extracellular signal-regulated kinases; PTEN: Phosphatase and tensin homolog; PI3K: Phosphatidylinositide-3-kinases; AKT: Protein Kinase B (PKB); mTOR: Mammalian target of rapamycin.

for colorectal neoplasia while the E1317Q one was associated with a significantly elevated adenoma risk. This meta-analysis may provide genetic insight into possible strategies for the prevention of colorectal neoplasia[37].

A very recent study, applying a comprehensive approach to systematically evaluate the tag single-nucleotide polymorphisms (tSNPs) in two key genes of the Wnt pathway, *APC* and *CTNNB1*, identified, by survival tree analysis, a higher-order genetic interaction profile consisting of the *APC* rs565453, *CTNNB1* rs2293303 and *APC* rs1816769 and this was significantly associated with overall survival; these SNPs might influence APC/CTNNB1 splicing and expression by altering the consensus splicing site sequences, the transposable elements, and the transcription factor binding sites. If validated, these biomarkers might be valuable to facilitate the identification of good treatment[8].

Recently, it was found that, in advanced-stage cancer, patients with *APC* mutation/high miR-21, an activator of the Wnt signaling pathway, had poorer overall survival so *APC* mutation and miR-21 expression could be used to predict the clinical outcome of CRC[38].

KRAS gene

The status of *KRAS* is generally accepted as a predictive marker for response to established EGFR inhibitors used for CRC because mutant *KRAS* is associated with resistance to anti-EGFR monoclonal antibody (mAb) immunotherapy with agents such as centuximab or panitumumab (Figure 1). It is the only established biomarker in clinical practice for CRC. The *KRAS* encodes a 21-kD protein (p21ras) involved in the G-protein signal transduction pathway, modulating cellular proliferation and differentiation. *KRAS* abnormalities are one of the earliest events in the stepwise progression of colorectal neoplasms, being detectable even in histologically unremarkable epithelium and aberrant crypt foci adjacent to cancers. Mutations of the *KRAS* oncogene result in constitutive activation of this signal transduction pathway and, consequently, unregulated proliferation and impaired differentiation[39]. The KRAS wild-type (WT) protein is transiently activated during tightly regulated signal transduction events. The binding of mAbs to EGFR normally induces receptor internalization, causing a direct inhibition of tyrosine kinase activity and the blockage of downstream RAS/RAF/MAPK signalling (Figure 1). However, activating *KRAS* mutations result in a constitutively active GTP-bound protein which consequently renders the downstream pathway permanently "switched on" irrespective of the activation status of upstream receptors including EGFR. In such an instance, the binding of an anti-EGFR mAb to EGFR and the inhibition of ligand-mediated receptor activation will fail to elicit any pathway suppressive effects. This constitutive pathway activation leads to unregulated proliferation, impaired differentiation, and resistance to anti-EGFR therapies[40] (Figure 1). Up to 90% of activating mutations of *KRAS* are detected in codons 12 (82%-87%) and 13 (13%-18%), but less frequently in codons 61, 63 and 146 and they are generally observed as somatic mutations. The most common types of *KRAS* mutations in CRC are point mutations, particularly G > A transitions and G > T trans-

versions. The codons 12 and 13 code for two adjacent glycine residues located in the proximity of the catalytic site[41]. Specific *KRAS* mutations may be heterogeneous in their phenotype. For example, codon 12 mutations were associated with a mucinous phenothype of CRC. By contrast, CRCs associated with codon 13 mutations were rather non-mucinous, but were characterized as more aggressive tumours with a greater metastatic potential[42]. However, a recent study observed that patients with an isolated p.G12A mutation (no other *KRAS* mutations) had aggressive disease (stage Ⅲ or Ⅳ and extensive metastatic or recurrent disease)[43,44].

KRAS mutations have also emerged as a major predictor or resistance to anti-EGFR mAbs, as confirmed by small data sets[45-47], and both retrospective and prospective trials[48-51]. In these studies, patients with metastatic CRC harbouring *KRAS* mutations had no benefits from treatment with cetuximab or panitumumab either alone or in combination with standard chemotherapy. This discovery led to the first practical implementation of personalized medicine in metastatic CRC, and *KRAS* mutations can be considered a highly specific negative biomarker for benefit of anti-EGFR mAbs. However it is intriguingly now coming to light that not all *KRAS* mutations are equal in their biological characteristics and their impact on mediating EGFR resistance, and that not all *KRAS* mutations will confer resistance to EGFR inhibitor therapy, probably due to heterogeneity of tumours[52-54].

Other clinicopathological and prognostic biomarkers of CRC

BRAF (V-raf murine sarcoma viral oncogenes homolog B1) is a member of the *RAF* gene family, it encodes a serine-threonine protein kinase, a downstream effector of activated RAS[55]. In the past decade, many studies have shown that *BRAF* somatic mutation presents in approximately 10% of CRCs[56,57]. A hotspot for *BRAF* mutation is the conversion of valine 600 to glutamic acid (V600E) within the kinase activation domain of the BRAF protein and this account for 80% of the *BRAF* mutations in CRC. This hot spot is suggested to be biologically distinct from other infrequent *BRAF* mutations, because the cancer cells having the V600E mutation can grow without functional RAS, and thus the *BRAF* V600E mutation has not been found in CRCs with *KRAS* mutations[58,59]. *BRAF* mutations have been linked with high grade, right side tumours, female gender, older age and MSI-H tumours[51]. A distinct pattern of metastatic spread has also been observed in *BRAF* mutant tumours, namely higher rates of peritoneal metastases, distant lymph node metastases and lower rates of lung metastases[60]. A very recent study demonstrated sex-related differences in the prognostic value of *BRAF* mutations in CRC, being particularly evident in men, in fact, *BRAF* mutation was associated with a significantly reduced cancer-specific survival in overall adjusted analysis[61].

Phosphatidylinositide-3-kinases (PI3K) are lipid kinases that promote various biological processes including cellular proliferation and survival. Mutations in the *PIK3CA* gene, which encodes the p110α catalytic subunit of PI3K, have been identified in many human solid tumours[62]. In colorectal cancers, *PIK3CA* mutations, which are found in 10%-20% of the cases, have been reported to be associated with specific clinicopathological features and molecular events, tumour proximal colonic location, mucinous differentiation, *KRAS* mutation, high levels CIMP and loss of *MGMT* expression[63]. It is unclear whether *PIK3CA* mutation defines a clinically and/or biologically relevant subset of tumours as there is significant overlap with *KRAS* and *BRAF* V600E mutation; a recent study observed that the adverse prognostic effect of *PIK3CA* mutation on survival was restricted to patients with a *BRAF* wild type tumour[63]. The majority of activating *PIK3CA* mutations map to three sites: exon 9, codons 542 and 545 in the helical domain, and exon 20, codon 1047 in the kinase domain. Mutation at any one of these sites has been shown to result in a gain of enzymatic function and to promote oncogenic transformation *in vitro* and *in vivo* (Figure 1). Co-existence of *PIK3CA* exon 9 and 20 mutations is associated with poor prognosis of CRC patients[64,65]. More recently, *PIK3CA* mutation was associated with longer survival in patients who use aspirin regularly after diagnosis[66].

Loss of *PTEN* expression (*PTEN* is a key tumour suppressor gene involved in the homeostatic maintenance of PI3K/AKT signalling) was associated with a higher rate of distant metastasis[67]. However, patients with PTEN expression had significantly longer overall survival than patients with *PTEN* loss tumour[68].

TP53 is a tumour suppressor gene encoding a protein involved in the regulation of cell division, growth arrest and apoptosis. A recent study[69] demonstrated that p53 expression was a significant prognostic factor for disease-free survival for the patients with stage Ⅲ tumour and also found stage Ⅲ tumours with wt-p53 high expression were associated with a significantly better prognosis after chemotherapy, according to previous findings[70].

A very recent study[71], exploring candidate tumour suppressor genes at chromosome 4q25-q28.2, found a novel candidate tumour suppressor gene, namely *NDST4*, identified at 4q26. This gene was markedly downregulated in CRC tumours and this genetic aberration was increased considerably in tumours with higher pathological stages (T3 and T4). NDST4 is one member of the N-deacetylase/N-sulfotransferase (heparan glucosaminyl) (NDST) family, which is responsible for heparan sulfate (HS) biosynthesis on a core protein to form heparan sulphate proteoglycans (HSPGs) that contribute to the tissue structure and function during development and adult homeostasis. The loss of function of *NDST4* might impair the modification of HS chains of specific HSPGs, leading to more invasive tumour cells through remodelling of the interaction of cell adhesion receptors and ligands. The genetic loss of *NDST4* might serve as a biomarker of adverse prognosis for patients with CRC[71].

Genetic biomarkers of response to treatment

During the last few years, chemotherapeutic agents, such as oxaliplatin, irinotecan, cetuximab, panitumumab, bevacizumab, aflibercept and regorafenib, have been approved as an addition to the traditional fluorouracil (5-FU) treatment, increasing the median overall survival. The survival of patients with metastatic CRC (mCRC) progressively improved over the past decades was due primarily to new chemotherapeutic combinations (5-FU, irinotecan, oxaliplatin) and the introduction of new therapies, among which there are two monoclonal antibodies against the receptor of epidermal growth factor receptor, cetuximab and panitumumab, effectual in the treatment of mCRC. However, these treatments are toxic and expensive, by this, the necessity to select patients most likely to have benefit with the treatment. Several analyses have revealed that patients with *KRAS* mutations receiving first and subsequent lines of treatment do not respond to cetuximab or panitumumab, and that they show no survival benefit from such treatments (Figure 1). Therefore, patients with mCRC with *KRAS* codon 12 or *KRAS* codon 13 mutated tumours are presently excluded from treatment with anti-EGFR mAb. Recent study has demonstrated, for the first time, that *KRAS* wt status is associated with better response to bevacizumab based chemotherapy and represents a positive prognostic factor for patients with advanced CRC treated in the first-line setting[72]. Codon 12 *KRAS* and *BRAF* mutations predict for adverse outcome of CRC patients receiving cetuximab[73]. *KRAS* mutations are, also, predictive of resistance to anti-EGFR antibodies when combined with irinotecan[49], response negatively affected also by *NRAS*, *BRAF* and *PI3KCA* mutations[43,74,75], as well as by a wild-type *TP53*[76]. So *KRAS* status has emerged as a major predictor of resistance to anti-EGFR mAb in the clinical setting but probably it is not the only to determine this type of resistance, in fact, a recent meta-analysis showed that *BRAF* mutation is associated with poor response to anti-EGFR mAbs and it is an adverse prognostic biomarker of the survival of patients with mCRC[77]. Regular aspirin use was associated with lower risk of *BRAF* wt colorectal cancer but not with *BRAF* mutated cancer risk. These findings suggest that *BRAF* mutant colon tumour cells may be less sensitive to the effect of aspirin[78]. In addition to *KRAS* and *BRAF* mutations, loss of *PTEN* expression and *PIK3CA* mutation is likely to be predictive of a lack of benefit to anti-EGFR therapy in metastatic colorectal cancer[79] (Figure 1); PTEN expressing tumours had statistically higher response rate for cetuximab based treatment than tumours with PTEN loss[68].

CRCs with MSI are reported to have a significantly better prognosis compared with CRCs without MSI (non-MSI), while MSI CRCs show resistance to 5-FU based chemotherapies. Although high frequency MSI tumours have better stage independent prognosis compared to those with CIN, MMR deficient CRC appears to be resistant to fluorouracil based treatment, but sensitive to other therapeutic regimens[80]. A summary of genetic biomarkers for CRC is shown in Table 1.

DNA METHYLATION BIOMARKERS IN CRC

Global DNA hypomethylation and depletion of overall 5-methylcytosine content in CRC tissues was observed for the first time in 1983, by Feinberg and Vogelstein. It was observed predominantly at CpG dinucleotides in repetitive sequences, occurring gradually, age-dependently, and early in carcinogenesis[81]. It was also clear from the beginning that global DNA hypomethylation in CRC tissues was accompanied by hypermethylation and transcriptional silencing of tumours suppressor genes or genes coding for DNA repair proteins[82]. Subsequent studies revealed that hundreds of genes are aberrantly methylated in the average CRC genome, and their number is ever-growing, including genes of the Wnt signalling pathway such as *APC*, *AXIN2*, *DKK1*, *SFRP1*, *SFRP2*, *WNT5A*, the DNA repair genes *MGMT,* h*MLH1*, and h*MLH2,* cell cycle-related genes such as $CDKN2A^{INK4a}$(*p14*), $CDKN2A^{A\text{-}INK4b}$(*p15*), and $CDKN2A^{ARF}$(*p16*), the RAS signalling genes *RASSF1A* and *RASSF1B*, and many more[11,83,84]. Although all CRCs are characterized by the presence of hypermethylation, a specific subgroup of them, denoted as the CpG island methylator phenotype (CIMP+), displays extensive levels of methylated genes[85]. By an epigenetic point of view, CRCs can be broadly divided into CIMP+ and non-CIMP tumours, but taking into account also genetic alterations, several subgroups have been proposed. For example, Hinoue and coworkers recently proposed the following four subtypes: (1) CIMP-high tumours exhibiting a very high frequency of cancer-specific DNA hypermethylation associated with *MLH1* methylation, microsatellite instability, and the *BRAF* V600E mutation; (2) CIMP-low tumours associated with *KRAS* mutations and characterized by methylation of a subset of CIMP-high associated genes; (3) Non-CIMP tumours characterized by *TP53* mutations and frequent occurrence in the distal colon; and (4) Non-CIMP tumours showing a low frequency of cancer-specific gene mutation and hypermethylation, and enriched of rectal tumours[7]. Increasing evidence suggests that several of those epigenetic modifications can be valuable biomarkers for CRC diagnosis, progression, prognosis, tendency to metastasis, and response to treatment (Table 2).

Methylation biomarkers of CRC diagnosis

Aberrant patterns of DNA methylation from CRC cells can be detected in tumours-derived cell-free DNA found in blood or feces of cancer patients, and there is also evidence that often DNA methylation profiles in blood reflect those in CRC tissues[86]. This led researchers to search for DNA methylation biomarkers in those specimens and to develop blood-based and stool-based non-invasive and cost-effective epigenetic CRC diagnostic tools[87]. The presence of aberrantly methylated septin 9

Table 1 Examples of genetic biomarkers for colorectal cancer

Biomarkers		Ref.
Genetic biomarkers	Prognosis	
Breakpoints of 17p11.2	Found in primary colonic tumours in CRC patients with liver metastasis	[22]
Loss of 18q	Poor prognosis	[23,24]
Loss of *SMAD*	Advanced stage disease (Ⅲ), lymph node metastases, shorter overall survival	[26]
APC mutations	Poorer overall survival	[39]
KRAS mutations	Heterogeneous phenotype of CRC	[43,44,45]
BRAF mutations	Specific phenotype and metastasis	[61,62]
PIK3CA mutations	Poor prognosis and specific clinicopathological features	[64]
Loss of *PTEN*	High rate of distant metastasis	[68]
TP53 expression	Worse prognosis	[70,71]
Loss of *NDST4*	Adverse prognosis	[72]
Candidate biomarkers	Chemoresistance/Chemosensitivity	
Loss of SMAD4	Poorer response to 5-FU	[28]
MSI	Resistance to 5-FU	[83]
KRAS, *BRAF*, *PI3KCA*, *PTEN* mutations	Resistance to anti-EGFR mAb	[46-52,74,75,79-81]

CRC: Colorectal cancer; mAb: Monoclonal antibodies; 5-FU: 5-fluorouracil.

(*SEPT9*) in plasma is a valuable and minimally invasive blood-based PCR test (Figure 2), showing a sensitivity and a specificity of almost 90% in the detection of CRC[13,87,88], and represents a currently commercialized test as it is able to detect CRC at all stages and locations[89]. Researchers have evaluated the possibility to include the methylation analysis of additional genes, such as for example *ALX4* and *HLTF*, to increase the sensitivity of this blood-based test[90,91]. Others are searching for different blood-based biomarkers than *SEPT9*. For example, the methylation status of secreted frizzled-related protein 2 gene (*SFRP2*) in CRC tissues, serum and fecal DNA was able to detect almost 67% CRCs[92], and recent genome-scale search of DNA-methylation biomarkers for blood-based detection of CRC revealed that methylated thrombomodulin (*THBD*) gene detects 74% of stage Ⅰ / Ⅱ CRCs at a specificity of 80%[93], and that methylation of the syndecan 2 (*SDC2*) gene has a sensitivity of 92% for stage Ⅰ CRC[94].

A stool-based test for the methylation analysis of the vimentin (*VIM*) gene is available in the United States (Figure 2) and has a specificity and sensitivity of almost 80%[14]. Several hypermethylated genes isolated from stool samples have been utilised as biomarkers for the detection of CRC or colorectal adenomas, including *APC*, *p16*, h*MLH1*, *MGMT*, *SFRP1*, *SFRP2* and *VIM*[95]. Two meta-analyses of those studies revealed that the sensitivity for the detection of CRC or adenomas ranged from 62% to 75%[95,96]. Recently, hypermethylation of fibrillin-1 (*FBN1*) was detected in CRC stool samples, and showed 72% sensitivity and 93% specificity for detecting CRC[97].

DNA methylation biomarkers of CRC staging and prognosis

A few years ago, Lao and Grady reviewed the genes that seem to be more commonly methylated in the multistep process leading from normal colonic epithelium to adenocarcinoma. At least six genes (*SLC5A8*, *SFRP1*, *SFRP2*, *CDH13*, *CRBP1*, and *RUNX3*) and two loci (*MINT1* and *MINT31*) have been consistently found to be methylated in the passage from a normal colon epithelium to an aberrant crypt focus. Other genes (*p14*, *HLTF*, *ITGA4*, *p16*, *CDH1* and *ESR1*) resulted frequently methylated in the passage from an aberrant crypt focus to polyp/adenoma, and four additional genes (*TIMP3*, *CXCL12*, *ID4*, and *IRF8*) could have a role in CRC progression and metastasis[11]. More recent studies revealed additional epigenetic biomarkers linked to CRC staging and progression. A high degree of LINE-1 hypomethylation was found in early-onset CRC, a clinically distinct form of CRC that is often associated with a poor prognosis[98]. LINE-1 hypomethylation leads to the activation of proto-oncogenes in CRC metastasis[99]. There is also indication that the clinical outcome of MSI CRCs depends on LINE-1 methylation, suggesting that lower LINE-1 methylation status serves as a significant prognostic parameter of adverse prognosis[100]. Serrated adenomas form a distinct subtype of colorectal premalignant lesions that may progress to malignancy along a different molecular pathway than the conventional adenoma-carcinoma pathway, and loss of expression of the slit homolog 2 (*SLIT2*) gene by promoter hypermethylation and loss of heterozygosity events are significantly associated with serrated adenoma development[101]. Methylation of the *WNT5A* gene, a member of the *WNT* gene family, has been frequently detected in early gastric carcinomas[102]. Also somatic mutations, allele loss, and DNA methylation of the cub and sushi multiple domains 1 (*CSMD1*) gene, whose function is still unclear, correlate with earlier clinical presentation in CRC[103].

Concerning CRC prognostic biomarkers, it was shown that DNA methylation of *p14*, *RASSF1A* and *APC* genes, defines a poor prognosis subset of CRC patients independently of both tumour stage and differentiation[104], whilst *MGMT* methylation seemed to play a protective role[104], and *MLH1* inactivation through hypermethylation was found to be related to improved survival[105]. A meta-analysis of 11 studies indicated that *p16*

Table 2 Examples of DNA methylation biomarkers for colorectal cancer diagnosis, progression, prognosis and treatment

DNA methylation biomarkers		Ref.
Methylated genes/loci	Frequently methylated in	
SLC5A8, *SFRP1*, *SFRP2*, *CDH13*, *CRABP1*, *RUNX3*, *MINT1*, *MINT31*, *WNT5A*	Normal colon epithelium → aberrant crypt focus	[11,105]
p14, *HLTF*, *ITGA4*, *CDKN2A/p16*, *CDH1*, *ESR1*	Aberrant crypt focus → polyp/adenoma	[11]
TIMP3, *CXCL12*, *ID4*, *IRF8*, *MGMT*, *hMLH1*	Polyp/adenoma → metastasis	[11]
SPARC, miR-34b/c, miR-126, miR-128	Lymphovascular invasion, metastasis	[114-117]
Methylation biomarkers	CRC Diagnosis	
SEPT9, *SFRP2*, *THDB*, *SBC2*	Blood-based PCR test for the detection of CRC	[91-97]
VIM, *FBN1*	Stool-based test for the detection of CRC	[14,100]
Methylation biomarkers	Prognosis	
p14, *RASSF1A*, and *APC*	Poor prognosis	[107]
MGMT, *hMLH1*	Improved survival	[107,108]
p16	Poor prognosis	[109]
HOPX-β	Worse prognosis of stage Ⅲ CRC	[110]
Extracellular matrix genes (*IGFBP3*, *EVL*, *CD109* and *FLNC*)	Worse survival	[111]
IGF2 hypomethylation	Poor prognosis, short survival	[112]
Polycomb genes (*SFRP1*, *MYOD1*, *HIC1*, and *SLIT2*)	Favourable prognosis in non-CIMP male patients	[113]
miR-34b/c, miR-126, miR-128	Invasive tumors	[115-117]
Candidate biomarkers	Chemoresistance/Chemosensitivity	
TFAP2E	No responsiveness to 5-FU, irinotecan, oxaliplatin	[118]
DYPD, *TYMP*, *UMPK*, *SPARC*	Their methylation might affect 5-FU treatment[1]	[16,119]
UGT1A1	Its methylation might affect irinotecan treatment[1]	[119]
MGMT	Clinical response to dacarbazine is restricted to those with MGMT hypermethylation	[120]

[1]Suggested biomarkers from cell culture studies, with limited or no evidence in humans. CRC: Colorectal cancer; miR: micro-RNA; PCR: Polymerase chain reaction; 5-FU: 5-fluorouracil.

hypermethylation might be a predictive factor for unfavourable prognosis of CRC patients[106]. Homeodomain-only protein X-β gene (*HOPX-β*) promoter methylation was recently shown to be frequent in human cancers and was suggested to act as a tumours suppressor gene. Particularly, *HOPX-β* promoter methylation was associated with worse prognosis of stage Ⅲ CRC patients and also with poor differentiation[107]. Methylation of genes in the extracellular matrix (ECM) remodelling pathway, such as *IGFBP3*, *EVL*, *CD109* and *FLNC*, was associated with worse survival, suggesting that methylation of this pathway might represent a prognostic signature in CRC[108]. Similarly, hypomethylation of the insulin growth factor Ⅱ (*IGF2*) differentially methylated region in colorectal tumours was associated with poor prognosis[109]. Conversely, methylation of the polycomb group target genes, including *SFRP1*, *MYOD1*, *HIC1*, and *SLIT2*, resulted in favourable prognosis in non-CIMP male patients[110].

Lymphovascular invasion of CRC was related to methylation of the gene encoding the secreted protein acidic and rich in cysteine (*SPARC*) in stromal cells[111]. Others analysed DNA methylation in mucosal wash fluid from patients undergoing colonoscopy, observing that methylation of the micro-RNA (miR-34b/c) had the greatest correlation with invasive tumours[112]. Methylation of miR-128 in CRC samples led to an upregulation of its target gene *NEK2* that resulted in lymphatic invasion and peritoneal dissemination[113]. It was also shown that epigenetic silencing of miR-126 contributes to tumour invasion and angiogenesis in CRC[114].

DNA methylation biomarkers and CRC chemotherapy

Epigenetic signatures in CRC are also of interest for their possible interactions with chemotherapeutic agents. Indeed, the epigenetic silencing of a particular gene might result in chemosensitivity or chemoresistance toward a particular therapeutic agent[16]. Crea *et al*[16] proposed a panel of genes whose aberrant methylation could contribute to chemosensitivity or chemoresistance to 5-FU, irinotecan, and oxaliplatin, three of the most frequently used drugs in CRC treatment. 5-FU antitumor activity is mainly exerted by inhibiting thymidylate synthase, in the *de novo* synthesis of pyrimidines. Increased *TYMS* expression is one of the major mechanisms of 5-FU chemoresistance, and there is indication that histone acetylation/deacetylation processes, rather than DNA methylation of the promoter, might be of relevance in epigenetically regulating *TYMS* expression in CRC. Several other genes that participate in pyrimidine metabolism might represent potential molecular determinants of 5-FU chemoresistance, including dihydropyrimidine dehydrogenase (*DYPD*), thymidine phosphorylase (*TYMP*), and uridine monophosphate/cytidine monophosphate kinase (*UMPK*) genes. Their potential epigenetic contribution to 5-FU resistance in CRC patients is under investigation[16].

Hypermethylation of the gene encoding the transcription factor AP-2 epsilon (*TFAP2E*) was found in 51% of CRC patients and resulted in clinical nonresponsiveness to chemotherapy (5-FU, irinotecan or oxaliplatin)[115]. Functional assays showed that *TFAP2E* chemoresistance is mediated through its downstream target gene *DKK4*,

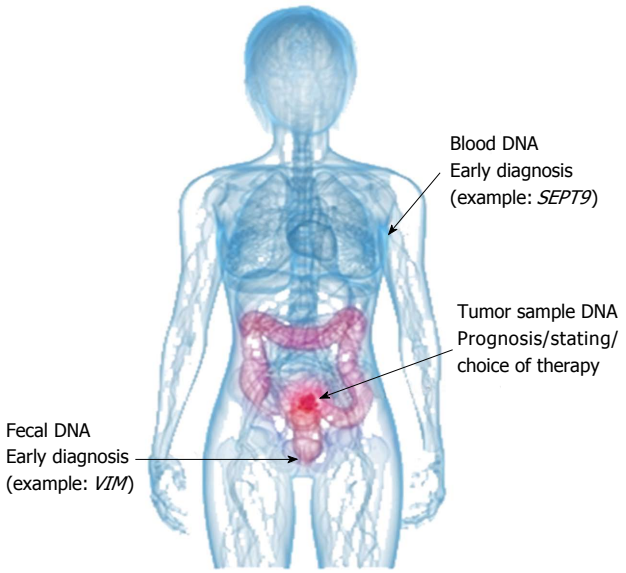


Figure 2 Sources of epigenetic biomarkers of colorectal cancer. Blood and fecal DNA samples represent available non-invasive sources of epigenetic diagnostic biomarkers of colorectal cancer, such as the analysis of methylation of septin 9 (*SEPT9*) and vimentin (*VIM*) genes, respectively. In addition, the analysis of DNA samples from bioptic tumor tissues could be of relevance for the best choice of therapeutic intervention.

encoding for dickkopf homolog 4 protein[115].

Other genes whose methylation might be associated with decreased sensitivity to 5-FU or irinotecan chemotherapy are *SPARC* coding for the matricellular protein osteonectin[116], and *UGT1A1* coding for the UDP glucuronosyltransferase- 1A1 enzyme, the major enzyme involved in irinotecan detoxification[16].

CRC patients who had failed standard therapies (oxaliplatin, irinotecan, 5-FU, or cetuximab or panitumumab if *KRAS* wild type) were treated with dacarbazine, an alkylating agent that exerts its antitumor activity inducing base pair mismatches[117]. Hypermethylation of the *MGMT* gene occurs in almost 40% of CRC patients, and clinical responses to dacarbazine are confined to those tumours harbouring epigenetic silencing of the *MGMT* gene[118].

FUTURE PERSPECTIVES

In addition to genetic aberrations, DNA methylation also plays important roles in the development of CRC. Recent genome-scale approaches revealed that CRCs exhibit multiple genetic alterations, including allelic imbalances (copy number alterations) at various chromosomal loci. For example, alongside with mutations of *TP53*, *KRAS*, *BRAF*, and *PIK3CA*, genomic losses commonly occurred at 3q26.1, 4q13.2, 6q21.32, 7q34, 8p12-23.3, 15qcen and 18, while gains were commonly found at 1q21.3-23.1, 7p22.3-q34, 13q12.11-14.11, and 20. Moreover, the total number of copy number alterations were significantly associated with the aberrant DNA methylation of six marker genes[118]. Similarly, transcriptome analyses are revealing thousands of genes whose expression is altered, likely through promoter methylation, in CRC tissues[119]. Goal of present and future research is to identify those biomarkers that could allow a feasible, cost-effective and non-invasive diagnosis of CRC, as well as to understand which panel of biomarkers can be used to better define patient's prognosis and the best choice of available treatments (Figure 2). Several examples are provided within this review suggesting the need to combine genetic and epigenetic data for a better diagnostic, prognostic and therapeutic approach. Integration of those data with transcriptome and proteome profiles could represent a valuable strategy to further understand the molecular pathways involved in CRC, as well as to improve life expectancies and quality of life of the patients.

REFERENCES

1 **Dawson MA**, Kouzarides T. Cancer epigenetics: from mechanism to therapy. *Cell* 2012; **150**: 12-27 [PMID: 22770212 DOI: 10.1016/j.cell.2012.06.013]
2 **Toyota M**, Suzuki H. Epigenetic drivers of genetic alterations. *Adv Genet* 2010; **70**: 309-323 [PMID: 20920753 DOI: 10.1016/B978-0-12-380866-0.60011-3]
3 **You JS**, Jones PA. Cancer genetics and epigenetics: two sides of the same coin? *Cancer Cell* 2012; **22**: 9-20 [PMID: 22789535 DOI: 10.1016/j.ccr.2012.06.008]
4 **Harrison S**, Benziger H. The molecular biology of colorectal carcinoma and its implications: a review. *Surgeon* 2011; **9**: 200-210 [PMID: 21672660 DOI: 10.1016/j.surge.2011.01.011]
5 **Migliore L**, Migheli F, Spisni R, Coppedè F. Genetics, cytogenetics, and epigenetics of colorectal cancer. *J Biomed Biotechnol* 2011; **2011**: 792362 [PMID: 21490705 DOI: 10.1155/2011/792362]
6 **Shen L**, Toyota M, Kondo Y, Lin E, Zhang L, Guo Y, Hernandez NS, Chen X, Ahmed S, Konishi K, Hamilton SR, Issa JP. Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. *Proc Natl Acad Sci USA* 2007; **104**: 18654-18659 [PMID: 18003927]
7 **Hinoue T**, Weisenberger DJ, Lange CP, Shen H, Byun HM, Van Den Berg D, Malik S, Pan F, Noushmehr H, van Dijk CM, Tollenaar RA, Laird PW. Genome-scale analysis of aberrant DNA methylation in colorectal cancer. *Genome Res* 2012; **22**: 271-282 [PMID: 21659424 DOI: 10.1101/gr.117523.110]
8 **Ting WC**, Chen LM, Pao JB, Yang YP, You BJ, Chang TY, Lan YH, Lee HZ, Bao BY. Common genetic variants in Wnt signaling pathway genes as potential prognostic biomarkers for colorectal cancer. *PLoS One* 2013; **8**: e56196 [PMID: 23405266 DOI: 10.1371/journal.pone.0056196]
9 **Lech G**, Slotwinski R, Krasnodebski IW. The role of tumor markers and biomarkers in colorectal cancer. *Neoplasma* 2013 Sep 20; Epub ahead of print [PMID: 24050544 DOI: 10.4149/neo_2014_003]
10 **Yokota T**. Are KRAS/BRAF mutations potent prognostic and/or predictive biomarkers in colorectal cancers? *Anticancer Agents Med Chem* 2012; **12**: 163-171 [PMID: 22043994 DOI: 10.2174/187152012799014968]
11 **Lao VV**, Grady WM. Epigenetics and colorectal cancer. *Nat Rev Gastroenterol Hepatol* 2011; **8**: 686-700 [PMID: 22009203 DOI: 10.1038/nrgastro.2011.173]
12 **Heyn H**, Esteller M. DNA methylation profiling in the clinic: applications and challenges. *Nat Rev Genet* 2012; **13**: 679-692 [PMID: 22945394 DOI: 10.1038/nrg3270]
13 **Grützmann R**, Molnar B, Pilarsky C, Habermann JK, Schlag PM, Saeger HD, Miehlke S, Stolz T, Model F, Roblick UJ, Bruch HP, Koch R, Liebenberg V, Devos T, Song X, Day RH, Sledziewski AZ, Lofton-Day C. Sensitive detection of colorectal cancer in peripheral blood by septin 9 DNA

methylation assay. *PLoS One* 2008; **3**: e3759 [PMID: 19018278 DOI: 10.1371/journal.pone.0003759]
14 **Itzkowitz S**, Brand R, Jandorf L, Durkee K, Millholland J, Rabeneck L, Schroy PC, Sontag S, Johnson D, Markowitz S, Paszat L, Berger BM. A simplified, noninvasive stool DNA test for colorectal cancer detection. *Am J Gastroenterol* 2008; **103**: 2862-2870 [PMID: 18759824 DOI: 10.1111/j.1572-0241.2008.02088.x]
15 **de Caestecker MP**, Hall CL, MacIver AG. Atypical antiglomerular basement membrane disease associated with thin membrane nephropathy. *Nephrol Dial Transplant* 1990; **5**: 909-913 [PMID: 2127824 DOI: 10.1158/1078-0432.CCR-10-2509]
16 **Crea F**, Nobili S, Paolicchi E, Perrone G, Napoli C, Landini I, Danesi R, Mini E. Epigenetics and chemoresistance in colorectal cancer: an opportunity for treatment tailoring and novel therapeutic strategies. *Drug Resist Updat* 2011; **14**: 280-296 [PMID: 21955833]
17 **Fearon ER**, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; **61**: 759-767 [PMID: 2188735]
18 **Ashktorab H**, Schäffer AA, Daremipouran M, Smoot DT, Lee E, Brim H. Distinct genetic alterations in colorectal cancer. *PLoS One* 2010; **5**: e8879 [PMID: 20126641 DOI: 10.1371/journal.pone.0008879]
19 **Bruin SC**, Klijn C, Liefers GJ, Braaf LM, Joosse SA, van Beers EH, Verwaal VJ, Morreau H, Wessels LF, van Velthuysen ML, Tollenaar RA, Van't Veer LJ. Specific genomic aberrations in primary colorectal cancer are associated with liver metastases. *BMC Cancer* 2010; **10**: 662 [PMID: 21126340 DOI: 10.1186/1471-2407-10-662]
20 **Nakao M**, Kawauchi S, Uchiyama T, Adachi J, Ito H, Chochi Y, Furuya T, Oga A, Sasaki K. DNA copy number aberrations associated with the clinicopathological features of colorectal cancers: Identification of genomic biomarkers by array-based comparative genomic hybridization. *Oncol Rep* 2011; **25**: 1603-1611 [PMID: 21468551 DOI: 10.3892/or.2011.1246]
21 **Muñoz-Bellvis L**, Fontanillo C, González-González M, Garcia E, Iglesias M, Esteban C, Gutierrez ML, Abad MM, Bengoechea O, De Las Rivas J, Orfao A, Sayagués JM. Unique genetic profile of sporadic colorectal cancer liver metastasis versus primary tumors as defined by high-density single-nucleotide polymorphism arrays. *Mod Pathol* 2012; **25**: 590-601 [PMID: 22222638 DOI: 10.1038/modpathol.2011.195]
22 **González-González M**, Muñoz-Bellvis L, Mackintosh C, Fontanillo C, Gutiérrez ML, Abad MM, Bengoechea O, Teodosio C, Fonseca E, Fuentes M, De Las Rivas J, Orfao A, Sayagués JM. Prognostic Impact of del(17p) and del(22q) as assessed by interphase FISH in sporadic colorectal carcinomas. *PLoS One* 2012; **7**: e42683 [PMID: 22912721 DOI: 10.1371/journal.pone.0042683]
23 **Fleming NI**, Jorissen RN, Mouradov D, Christie M, Sakthianandeswaren A, Palmieri M, Day F, Li S, Tsui C, Lipton L, Desai J, Jones IT, McLaughlin S, Ward RL, Hawkins NJ, Ruszkiewicz AR, Moore J, Zhu HJ, Mariadason JM, Burgess AW, Busam D, Zhao Q, Strausberg RL, Gibbs P, Sieber OM. SMAD2, SMAD3 and SMAD4 mutations in colorectal cancer. *Cancer Res* 2013; **73**: 725-735 [PMID: 23139211 DOI: 10.1158/0008-5472]
24 **Watanabe T**, Kobunai T, Yamamoto Y, Konishi T, Yano H, Iinuma H, Hayama T, Nozawa K, Ishihara S, Matsuda K. Prognostic significance of 18q loss of heterzygosity in microsatellite-stable colorectal cancer. *J Clin Oncol* 2010; **28**: e119; author reply e120 [PMID: 20124161 DOI: 10.1200/JCO.2009.27.0256]
25 **Zhang B**, Halder SK, Kashikar ND, Cho YJ, Datta A, Gorden DL, Datta PK. Antimetastatic role of Smad4 signaling in colorectal cancer. *Gastroenterology* 2010; **138**: 969-80.e1-3 [PMID: 19909744 DOI: 10.1053/j.gastro.2009.11.004]
26 **Roth AD**, Delorenzi M, Tejpar S, Yan P, Klingbiel D, Fiocca R, d'Ario G, Cisar L, Labianca R, Cunningham D, Nordlinger B, Bosman F, Van Cutsem E. Integrated analysis of molecular and clinical prognostic factors in stage II/III colon cancer. *J Natl Cancer Inst* 2012; **104**: 1635-1646 [PMID: 23104212 DOI: 10.1093/jnci/djs427]
27 **Kolesnikov GF**, Pirogov VA, Saĭdakova NA. [Clinical characteristics and bioelectrical activity of the muscles of the urogenital apparatus in patients with enuresis]. *Pediatriia* 1975; (**12**): 29-30 [PMID: 1223777]
28 **Alhopuro P**, Alazzouzi H, Sammalkorpi H, Dávalos V, Salovaara R, Hemminki A, Järvinen H, Mecklin JP, Schwartz S, Aaltonen LA, Arango D. SMAD4 levels and response to 5-fluorouracil in colorectal cancer. *Clin Cancer Res* 2005; **11**: 6311-6316 [PMID: 16144935]
29 **Walther A**, Houlston R, Tomlinson I. Association between chromosomal instability and prognosis in colorectal cancer: a meta-analysis. *Gut* 2008; **57**: 941-950 [PMID: 18364437 DOI: 10.1136/gut.2007.135004]
30 **Grady WM**, Carethers JM. Genomic and epigenetic instability in colorectal cancer pathogenesis. *Gastroenterology* 2008; **135**: 1079-1099 [PMID: 18773902 DOI: 10.1053/j.gastro.2008.07.076]
31 **Benatti P**, Gafà R, Barana D, Marino M, Scarselli A, Pedroni M, Maestri I, Guerzoni L, Roncucci L, Menigatti M, Roncari B, Maffei S, Rossi G, Ponti G, Santini A, Losi L, Di Gregorio C, Oliani C, Ponz de Leon M, Lanza G. Microsatellite instability and colorectal cancer prognosis. *Clin Cancer Res* 2005; **11**: 8332-8340 [PMID: 16322293]
32 **Kloor M**, Staffa L, Ahadova A, von Knebel Doeberitz M. Clinical significance of microsatellite instability in colorectal cancer. *Langenbecks Arch Surg* 2014; **399**: 23-31 [PMID: 24048684]
33 **Popat S**, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. *J Clin Oncol* 2005; **23**: 609-618 [PMID: 15659508]
34 **Sargent DJ**, Marsoni S, Monges G, Thibodeau SN, Labianca R, Hamilton SR, French AJ, Kabat B, Foster NR, Torri V, Ribic C, Grothey A, Moore M, Zaniboni A, Seitz JF, Sinicrope F, Gallinger S. Defective mismatch repair as a predictive marker for lack of efficacy of fluorouracil-based adjuvant therapy in colon cancer. *J Clin Oncol* 2010; **28**: 3219-3226 [PMID: 20498393 DOI: 10.1200/JCO.2009.27.1825]
35 **Sinicrope FA**, Foster NR, Thibodeau SN, Marsoni S, Monges G, Labianca R, Kim GP, Yothers G, Allegra C, Moore MJ, Gallinger S, Sargent DJ. DNA mismatch repair status and colon cancer recurrence and survival in clinical trials of 5-fluorouracil-based adjuvant therapy. *J Natl Cancer Inst* 2011; **103**: 863-875 [PMID: 21597022 DOI: 10.1093/jnci/djr153]
36 **Losso GM**, Moraes Rda S, Gentili AC, Messias-Reason IT. Microsatellite instability--MSI markers (BAT26, BAT25, D2S123, D5S346, D17S250) in rectal cancer. *Arq Bras Cir Dig* 2012; **25**: 240-244 [PMID: 23411922]
37 **Liang J**, Lin C, Hu F, Wang F, Zhu L, Yao X, Wang Y, Zhao Y. APC polymorphisms and the risk of colorectal neoplasia: a HuGE review and meta-analysis. *Am J Epidemiol* 2013; **177**: 1169-1179 [PMID: 23576677 DOI: 10.1093/aje/kws382]
38 **Chen TH**, Chang SW, Huang CC, Wang KL, Yeh KT, Liu CN, Lee H, Lin CC, Cheng YW. The prognostic significance of APC gene mutation and miR-21 expression in advanced-stage colorectal cancer. *Colorectal Dis* 2013; **15**: 1367-1374 [PMID: 23773491 DOI: 10.1111/codi.12318]
39 **Bolocan A**, Ion D, Ciocan DN, Paduraru DN. Prognostic and predictive factors in colorectal cancer. *Chirurgia* (Bucur) 2012; **107**: 555-563 [PMID: 23116828]
40 **Scaltriti M**, Baselga J. The epidermal growth factor receptor pathway: a model for targeted therapy. *Clin Cancer Res* 2006; **12**: 5268-5272 [PMID: 17000658]
41 **Bazan V**, Migliavacca M, Zanna I, Tubiolo C, Grassi N, Latteri MA, La Farina M, Albanese I, Dardanoni G, Salerno

S, Tomasino RM, Labianca R, Gebbia N, Russo A. Specific codon 13 K-ras mutations are predictive of clinical outcome in colorectal cancer patients, whereas codon 12 K-ras mutations are associated with mucinous histotype. *Ann Oncol* 2002; **13**: 1438-1446 [PMID: 12196370]

42 **Bazan V**, Agnese V, Corsale S, Calò V, Valerio MR, Latteri MA, Vieni S, Grassi N, Cicero G, Dardanoni G, Tomasino RM, Colucci G, Gebbia N, Russo A. Specific TP53 and/or Ki-ras mutations as independent predictors of clinical outcome in sporadic colorectal adenocarcinomas: results of a 5-year Gruppo Oncologico dell'Italia Meridionale (GOIM) prospective study. *Ann Oncol* 2005; **16** Suppl 4: iv50-iv55 [PMID: 15923430]

43 **De Roock W**, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, Kalogeras KT, Kotoula V, Papamichael D, Laurent-Puig P, Penault-Llorca F, Rougier P, Vincenzi B, Santini D, Tonini G, Cappuzzo F, Frattini M, Molinari F, Saletti P, De Dosso S, Martini M, Bardelli A, Siena S, Sartore-Bianchi A, Tabernero J, Macarulla T, Di Fiore F, Gangloff AO, Ciardiello F, Pfeiffer P, Qvortrup C, Hansen TP, Van Cutsem E, Piessevaux H, Lambrechts D, Delorenzi M, Tejpar S. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. *Lancet Oncol* 2010; **11**: 753-762 [PMID: 20619739 DOI: 10.1016/S1470-2045(10)70130-3]

44 **Cushman-Vokoun AM**, Stover DG, Zhao Z, Koehler EA, Berlin JD, Vnencak-Jones CL. Clinical utility of KRAS and BRAF mutations in a cohort of patients with colorectal neoplasms submitted for microsatellite instability testing. *Clin Colorectal Cancer* 2013; **12**: 168-178 [PMID: 23773459 DOI: 10.1016/j.clcc.2013.04.005]

45 **De Roock W**, Piessevaux H, De Schutter J, Janssens M, De Hertogh G, Personeni N, Biesmans B, Van Laethem JL, Peeters M, Humblet Y, Van Cutsem E, Tejpar S. KRAS wild-type state predicts survival and is associated to early radiological response in metastatic colorectal cancer treated with cetuximab. *Ann Oncol* 2008; **19**: 508-515 [PMID: 17998284]

46 **Sastre J**, Aranda E, Grávalos C, Massutí B, Varella-Garcia M, Rivera F, Soler G, Carrato A, Manzano JL, Díaz-Rubio E, Hidalgo M. First-line single-agent cetuximab in elderly patients with metastatic colorectal cancer. A phase II clinical and molecular study of the Spanish group for digestive tumor therapy (TTD). *Crit Rev Oncol Hematol* 2011; **77**: 78-84 [PMID: 20042346 DOI: 10.1016/j.critrevonc.2009.11.005]

47 **Soeda H**, Shimodaira H, Watanabe M, Suzuki T, Gamoh M, Mori T, Komine K, Iwama N, Kato S, Ishioka C. Clinical usefulness of KRAS, BRAF, and PIK3CA mutations as predictive markers of cetuximab efficacy in irinotecan- and oxaliplatin-refractory Japanese patients with metastatic colorectal cancer. *Int J Clin Oncol* 2013; **18**: 670-677 [PMID: 22638623 DOI: 10.1007/s10147-012-0422-8]

48 **Karapetis CS**, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, Simes RJ, Chalchal H, Shapiro JD, Robitaille S, Price TJ, Shepherd L, Au HJ, Langer C, Moore MJ, Zalcberg JR. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *N Engl J Med* 2008; **359**: 1757-1765 [PMID: 18946061 DOI: 10.1056/NEJMoa0804385]

49 **Peeters M**, Price TJ, Cervantes A, Sobrero AF, Ducreux M, Hotko Y, André T, Chan E, Lordick F, Punt CJ, Strickland AH, Wilson G, Ciuleanu TE, Roman L, Van Cutsem E, Tzekova V, Collins S, Oliner KS, Rong A, Gansert J. Randomized phase III study of panitumumab with fluorouracil, leucovorin, and irinotecan (FOLFIRI) compared with FOLFIRI alone as second-line treatment in patients with metastatic colorectal cancer. *J Clin Oncol* 2010; **28**: 4706-4713 [PMID: 20921462 DOI: 10.1200/JCO.2009.27.6055]

50 **Bokemeyer C**, Bondarenko I, Hartmann JT, de Braud F, Schuch G, Zubel A, Celik I, Schlichting M, Koralewski P. Efficacy according to biomarker status of cetuximab plus FOLFOX-4 as first-line treatment for metastatic colorectal cancer: the OPUS study. *Ann Oncol* 2011; **22**: 1535-1546 [PMID: 21228335 DOI: 10.1093/annonc/mdq632]

51 **Van Cutsem E**, Köhne CH, Láng I, Folprecht G, Nowacki MP, Cascinu S, Shchepotin I, Maurel J, Cunningham D, Tejpar S, Schlichting M, Zubel A, Celik I, Rougier P, Ciardiello F. Cetuximab plus irinotecan, fluorouracil, and leucovorin as first-line treatment for metastatic colorectal cancer: updated analysis of overall survival according to tumor KRAS and BRAF mutation status. *J Clin Oncol* 2011; **29**: 2011-2019 [PMID: 21502544 DOI: 10.1200/JCO.2010.33.5091]

52 **Tejpar S**, Celik I, Schlichting M, Sartorius U, Bokemeyer C, Van Cutsem E. Association of KRAS G13D tumor mutations with outcome in patients with metastatic colorectal cancer treated with first-line chemotherapy with or without cetuximab. *J Clin Oncol* 2012; **30**: 3570-3577 [PMID: 22734028 DOI: 10.1200/JCO.2012.42.2592]

53 **Chen J**, Ye Y, Sun H, Shi G. Association between KRAS codon 13 mutations and clinical response to anti-EGFR treatment in patients with metastatic colorectal cancer: results from a meta-analysis. *Cancer Chemother Pharmacol* 2013; **71**: 265-272 [PMID: 23090619 DOI: 10.1007/s00280-012-2005-9]

54 **Peeters M**, Douillard JY, Van Cutsem E, Siena S, Zhang K, Williams R, Wiezorek J. Mutant KRAS codon 12 and 13 alleles in patients with metastatic colorectal cancer: assessment as prognostic and predictive biomarkers of response to panitumumab. *J Clin Oncol* 2013; **31**: 759-765 [PMID: 23182985 DOI: 10.1200/JCO.2012.45.1492]

55 **Sridhar SS**, Hedley D, Siu LL. Raf kinase as a target for anticancer therapeutics. *Mol Cancer Ther* 2005; **4**: 677-685 [PMID: 15827342]

56 **Goel A**, Nagasaka T, Arnold CN, Inoue T, Hamilton C, Niedzwiecki D, Compton C, Mayer RJ, Goldberg R, Bertagnolli MM, Boland CR. The CpG island methylator phenotype and chromosomal instability are inversely correlated in sporadic colorectal cancer. *Gastroenterology* 2007; **132**: 127-138 [PMID: 17087942]

57 **Nagasaka T**, Koi M, Kloor M, Gebert J, Vilkin A, Nishida N, Shin SK, Sasamoto H, Tanaka N, Matsubara N, Boland CR, Goel A. Mutations in both KRAS and BRAF may contribute to the methylator phenotype in colon cancer. *Gastroenterology* 2008; **134**: 1950-1960, 1960.e1 [PMID: 18435933 DOI: 10.1053/j.gastro.2008.02.094]

58 **De Roock W**, Jonker DJ, Di Nicolantonio F, Sartore-Bianchi A, Tu D, Siena S, Lamba S, Arena S, Frattini M, Piessevaux H, Van Cutsem E, O'Callaghan CJ, Khambata-Ford S, Zalcberg JR, Simes J, Karapetis CS, Bardelli A, Tejpar S. Association of KRAS p.G13D mutation with outcome in patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab. *JAMA* 2010; **304**: 1812-1820 [PMID: 20978259 DOI: 10.1001/jama.2010.1535]

59 **Tol J**, Dijkstra JR, Klomp M, Teerenstra S, Dommerholt M, Vink-Börger ME, van Cleef PH, van Krieken JH, Punt CJ, Nagtegaal ID. Markers for EGFR pathway activation as predictor of outcome in metastatic colorectal cancer patients treated with or without cetuximab. *Eur J Cancer* 2010; **46**: 1997-2009 [PMID: 20413299 DOI: 10.1016/j.ejca.2010.03.036]

60 **Tran B**, Kopetz S, Tie J, Gibbs P, Jiang ZQ, Lieu CH, Agarwal A, Maru DM, Sieber O, Desai J. Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer. *Cancer* 2011; **117**: 4623-4632 [PMID: 21456008 DOI: 10.1002/cncr.26086]

61 **Wangefjord S**, Sundström M, Zendehrokh N, Lindquist KE, Nodin B, Jirström K, Eberhard J. Sex differences in the prognostic significance of KRAS codons 12 and 13, and BRAF mutations in colorectal cancer: a cohort study. *Biol Sex Differ* 2013; **4**: 17 [PMID: 24020794]

62 **Samuels Y**, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, Yan H, Gazdar A, Powell SM, Riggins GJ, Willson JK, Mar-

kowitz S, Kinzler KW, Vogelstein B, Velculescu VE. High frequency of mutations of the PIK3CA gene in human cancers. *Science* 2004; **304**: 554 [PMID: 15016963]

63 **Rosty C**, Young JP, Walsh MD, Clendenning M, Sanderson K, Walters RJ, Parry S, Jenkins MA, Win AK, Southey MC, Hopper JL, Giles GG, Williamson EJ, English DR, Buchanan DD. PIK3CA activating mutation in colorectal carcinoma: associations with molecular features and survival. *PLoS One* 2013; **8**: e65479 [PMID: 23785428 DOI: 10.1371/journal.pone.0065479]

64 **Zhao L**, Vogt PK. Helical domain and kinase domain mutations in p110alpha of phosphatidylinositol 3-kinase induce gain of function by different mechanisms. *Proc Natl Acad Sci USA* 2008; **105**: 2652-2657 [PMID: 18268322 DOI: 10.1073/pnas.0712169105]

65 **Liao X**, Morikawa T, Lochhead P, Imamura Y, Kuchiba A, Yamauchi M, Nosho K, Qian ZR, Nishihara R, Meyerhardt JA, Fuchs CS, Ogino S. Prognostic role of PIK3CA mutation in colorectal cancer: cohort study and literature review. *Clin Cancer Res* 2012; **18**: 2257-2268 [PMID: 22357840 DOI: 10.1158/1078-0432]

66 **Liao X**, Lochhead P, Nishihara R, Morikawa T, Kuchiba A, Yamauchi M, Imamura Y, Qian ZR, Baba Y, Shima K, Sun R, Nosho K, Meyerhardt JA, Giovannucci E, Fuchs CS, Chan AT, Ogino S. Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. *N Engl J Med* 2012; **367**: 1596-1606 [PMID: 23094721 DOI: 10.1056/NEJMoa1207756]

67 **Neumann J**, Wehweck L, Maatz S, Engel J, Kirchner T, Jung A. Alterations in the EGFR pathway coincide in colorectal cancer and impact on prognosis. *Virchows Arch* 2013; **463**: 509-523 [PMID: 23934607]

68 **Tural D**, Batur S, Erdamar S, Akar E, Kepil N, Mandel NM, Serdengeçti S. Analysis of PTEN, BRAF and PI3K status for determination of benefit from cetuximab therapy in metastatic colorectal cancer patients refractory to chemotherapy with wild-type KRAS. *Tumour Biol* 2013 Sep 1; Epub ahead of print [PMID: 23996432]

69 **Huh JW**, Kim HR, Kim YJ. Prognostic role of p53 messenger ribonucleic acid expression in patients after curative resection for stage I to III colorectal cancer: association with colon cancer stem cell markers. *J Am Coll Surg* 2013; **216**: 1063-1069 [PMID: 23571142 DOI: 10.1016/j.jamcollsurg.2013.01.058]

70 **Russo A**, Bazan V, Iacopetta B, Kerr D, Soussi T, Gebbia N. The TP53 colorectal cancer international collaborative study on the prognostic and predictive significance of p53 mutation: influence of tumor site, type of mutation, and adjuvant treatment. *J Clin Oncol* 2005; **23**: 7518-7528 [PMID: 16172461]

71 **Tzeng ST**, Tsai MH, Chen CL, Lee JX, Jao TM, Yu SL, Yen SJ, Yang YC. NDST4 is a novel candidate tumor suppressor gene at chromosome 4q26 and its genetic loss predicts adverse prognosis in colorectal cancer. *PLoS One* 2013; **8**: e67040 [PMID: 23825612 DOI: 10.1371/journal.pone.0067040]

72 **Petrelli F**, Coinu A, Cabiddu M, Ghilardi M, Barni S. KRAS as prognostic biomarker in metastatic colorectal cancer patients treated with bevacizumab: a pooled analysis of 12 published trials. *Med Oncol* 2013; **30**: 650 [PMID: 23828442 DOI: 10.1007/s12032-013-0650-4]

73 **Pentheroudakis G**, Kotoula V, De Roock W, Kouvatseas G, Papakostas P, Makatsoris T, Papamichael D, Xanthakis I, Sgouros J, Televantou D, Kafiri G, Tsamandas AC, Razis E, Galani E, Bafaloukos D, Efstratiou I, Bompolaki I, Pectasides D, Pavlidis N, Tejpar S, Fountzilas G. Biomarkers of benefit from cetuximab-based therapy in metastatic colorectal cancer: interaction of EGFR ligand expression with RAS/RAF, PIK3CA genotypes. *BMC Cancer* 2013; **13**: 49 [PMID: 23374602 DOI: 10.1186/1471-2407-13-49]

74 **Di Nicolantonio F**, Martini M, Molinari F, Sartore-Bianchi A, Arena S, Saletti P, De Dosso S, Mazzucchelli L, Frattini M, Siena S, Bardelli A. Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *J Clin Oncol* 2008; **26**: 5705-5712 [PMID: 19001320 DOI: 10.1200/JCO.2008.18.0786]

75 **Mao C**, Yang ZY, Hu XF, Chen Q, Tang JL. PIK3CA exon 20 mutations as a potential biomarker for resistance to anti-EGFR monoclonal antibodies in KRAS wild-type metastatic colorectal cancer: a systematic review and meta-analysis. *Ann Oncol* 2012; **23**: 1518-1525 [PMID: 22039088 DOI: 10.1093/annonc/mdr464]

76 **Oden-Gangloff A**, Di Fiore F, Bibeau F, Lamy A, Bougeard G, Charbonnier F, Blanchard F, Tougeron D, Ychou M, Boissière F, Le Pessot F, Sabourin JC, Tuech JJ, Michel P, Frebourg T. TP53 mutations predict disease control in metastatic colorectal cancer treated with cetuximab-based chemotherapy. *Br J Cancer* 2009; **100**: 1330-1335 [PMID: 19367287 DOI: 10.1038/sj.bjc.6605008]

77 **Xu Q**, Xu AT, Zhu MM, Tong JL, Xu XT, Ran ZH. Predictive and prognostic roles of BRAF mutation in patients with metastatic colorectal cancer treated with anti-epidermal growth factor receptor monoclonal antibodies: a meta-analysis. *J Dig Dis* 2013; **14**: 409-416 [PMID: 23615046 DOI: 10.1111/1751-2980.12063]

78 **Nishihara R**, Lochhead P, Kuchiba A, Jung S, Yamauchi M, Liao X, Imamura Y, Qian ZR, Morikawa T, Wang M, Spiegelman D, Cho E, Giovannucci E, Fuchs CS, Chan AT, Ogino S. Aspirin use and risk of colorectal cancer according to BRAF mutation status. *JAMA* 2013; **309**: 2563-2571 [PMID: 23800934 DOI: 10.1001/jama.2013.6599]

79 **Sood A**, McClain D, Maitra R, Basu-Mallick A, Seetharam R, Kaubisch A, Rajdev L, Mariadason JM, Tanaka K, Goel S. PTEN gene expression and mutations in the PIK3CA gene as predictors of clinical benefit to anti-epidermal growth factor receptor antibody therapy in patients with KRAS wild-type metastatic colorectal cancer. *Clin Colorectal Cancer* 2012; **11**: 143-150 [PMID: 22285706]

80 **Devaud N**, Gallinger S. Chemotherapy of MMR-deficient colorectal cancer. *Fam Cancer* 2013; **12**: 301-306 [PMID: 23539382 DOI: 10.1007/s10689-013-9633-z]

81 **Feinberg AP**, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* 1983; **301**: 89-92 [PMID: 6185846]

82 **Feinberg AP**, Vogelstein B. Hypomethylation of ras oncogenes in primary human cancers. *Biochem Biophys Res Commun* 1983; **111**: 47-54 [PMID: 6187346]

83 **Coppedè F**. Epigenetic biomarkers of colorectal cancer: Focus on DNA methylation. *Cancer Lett* 2014; **342**: 238-247 [PMID: 22202641]

84 **Goel A**, Boland CR. Epigenetics of colorectal cancer. *Gastroenterology* 2012; **143**: 1442-1460.e1 [PMID: 23000599 DOI: 10.1053/j.gastro.2012.09.032]

85 **Toyota M**, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci USA* 1999; **96**: 8681-8686 [PMID: 10411935]

86 **Danese E**, Minicozzi AM, Benati M, Montagnana M, Paviati E, Salvagno GL, Gusella M, Pasini F, Guidi GC, Lippi G. Epigenetic alteration: new insights moving from tissue to plasma - the example of PCDH10 promoter methylation in colorectal cancer. *Br J Cancer* 2013; **109**: 807-813 [PMID: 23839493 DOI: 10.1038/bjc.2013.351]

87 **Lange CP**, Laird PW. Clinical applications of DNA methylation biomarkers in colorectal cancer. *Epigenomics* 2013; **5**: 105-108 [PMID: 23566085 DOI: 10.2217/epi.13.4]

88 **deVos T**, Tetzner R, Model F, Weiss G, Schuster M, Distler J, Steiger KV, Grützmann R, Pilarsky C, Habermann JK, Fleshner PR, Oubre BM, Day R, Sledziewski AZ, Lofton-Day C. Circulating methylated SEPT9 DNA in plasma is a biomarker for colorectal cancer. *Clin Chem* 2009; **55**: 1337-1346 [PMID: 19406918 DOI: 10.1373/clinchem.2008.115808]

89 **Summers T**, Langan RC, Nissan A, Brücher BL, Bilchik AJ, Protic M, Daumer M, Avital I, Stojadinovic A. Serum-based

DNA methylation biomarkers in colorectal cancer: potential for screening and early detection. *J Cancer* 2013; **4**: 210-216 [PMID: 23459561 DOI: 10.7150/jca.5839]

90 **Tänzer M**, Balluff B, Distler J, Hale K, Leodolter A, Röcken C, Molnar B, Schmid R, Lofton-Day C, Schuster T, Ebert MP. Performance of epigenetic markers SEPT9 and ALX4 in plasma for detection of colorectal precancerous lesions. *PLoS One* 2010; **5**: e9061 [PMID: 20140221 DOI: 10.1371/journal.pone.0009061]

91 **Kostin PA**, Zakharzhevskaia NB, Generozov EV, Govorun VM, Chernyshov SV, Shchelygin IuA. [Hypermethylation of the CDH1, SEPT9, HLTF and ALX4 genes and their diagnostic significance in colorectal cancer]. *Vopr Onkol* 2010; **56**: 162-168 [PMID: 20552891]

92 **Tang D**, Liu J, Wang DR, Yu HF, Li YK, Zhang JQ. Diagnostic and prognostic value of the methylation status of secreted frizzled-related protein 2 in colorectal cancer. *Clin Invest Med* 2011; **34**: E88-E95 [PMID: 21463549]

93 **Lange CP**, Campan M, Hinoue T, Schmitz RF, van der Meulen-de Jong AE, Slingerland H, Kok PJ, van Dijk CM, Weisenberger DJ, Shen H, Tollenaar RA, Laird PW. Genome-scale discovery of DNA-methylation biomarkers for blood-based detection of colorectal cancer. *PLoS One* 2012; **7**: e50266 [PMID: 23209692 DOI: 10.1371/journal.pone.0050266]

94 **Oh T**, Kim N, Moon Y, Kim MS, Hoehn BD, Park CH, Kim TS, Kim NK, Chung HC, An S. Genome-wide identification and validation of a novel methylation biomarker, SDC2, for blood-based detection of colorectal cancer. *J Mol Diagn* 2013; **15**: 498-507 [PMID: 23747112 DOI: 10.1016/j.jmoldx.2013.03.004]

95 **Luo YX**, Chen DK, Song SX, Wang L, Wang JP. Aberrant methylation of genes in stool samples as diagnostic biomarkers for colorectal cancer or adenomas: a meta-analysis. *Int J Clin Pract* 2011; **65**: 1313-1320 [PMID: 22093539 DOI: 10.1111/j.1742-1241.2011.02800.x]

96 **Yang H**, Xia BQ, Jiang B, Wang G, Yang YP, Chen H, Li BS, Xu AG, Huang YB, Wang XY. Diagnostic value of stool DNA testing for multiple markers of colorectal cancer and advanced adenoma: a meta-analysis. *Can J Gastroenterol* 2013; **27**: 467-475 [PMID: 23936877]

97 **Guo Q**, Song Y, Zhang H, Wu X, Xia P, Dang C. Detection of hypermethylated fibrillin-1 in the stool samples of colorectal cancer patients. *Med Oncol* 2013; **30**: 695 [PMID: 23963856 DOI: 10.1007/s12032-013-0695-4]

98 **Antelo M**, Balaguer F, Shia J, Shen Y, Hur K, Moreira L, Cuatrecasas M, Bujanda L, Giraldez MD, Takahashi M, Cabanne A, Barugel ME, Arnold M, Roca EL, Andreu M, Castellvi-Bel S, Llor X, Jover R, Castells A, Boland CR, Goel A. A high degree of LINE-1 hypomethylation is a unique feature of early-onset colorectal cancer. *PLoS One* 2012; **7**: e45357 [PMID: 23049789 DOI: 10.1371/journal.pone.0045357]

99 **Hur K**, Cejas P, Feliu J, Moreno-Rubio J, Burgos E, Boland CR, Goel A. Hypomethylation of long interspersed nuclear element-1 (LINE-1) leads to activation of proto-oncogenes in human colorectal cancer metastasis. *Gut* 2013; Epub ahead of print [PMID: 23704319]

100 **Rhee YY**, Kim MJ, Bae JM, Koh JM, Cho NY, Juhnn YS, Kim D, Kang GH. Clinical outcomes of patients with microsatellite-unstable colorectal carcinomas depend on L1 methylation level. *Ann Surg Oncol* 2012; **19**: 3441-3448 [PMID: 22618722 DOI: 10.1245/s10434-012-2410-7]

101 **Kirvelä O**, Nieminen S. Treatment of painful neuromas with neurolytic blockade. *Pain* 1990; **41**: 161-165 [PMID: 2367142 DOI: 10.1371/journal.pgen.1003488]

102 **Hibi K**, Sakata M, Yokomizi K, Kitamura YH, Sakuraba K, Shirahata R, Goto T, Mizukami H, Saito M, Ishibashi K, Kigawa G, Nemoto H, Sanada Y. Methylation of the WNT5A gene is frequently detected in early gastric carcinoma. *Hepatogastroenterology* 2012; **59**: 2661-2663 [PMID: 23178630 DOI: 10.5754/hge09664]

103 **Shull AY**, Clendenning ML, Ghoshal-Gupta S, Farrell CL, Vangapandu HV, Dudas L, Wilkerson BJ, Buckhaults PJ. Somatic mutations, allele loss, and DNA methylation of the Cub and Sushi Multiple Domains 1 (CSMD1) gene reveals association with early age of diagnosis in colorectal cancer patients. *PLoS One* 2013; **8**: e58731 [PMID: 23505554 DOI: 10.1371/journal.pone.0058731]

104 **Nilsson TK**, Löf-Öhlin ZM, Sun XF. DNA methylation of the p14ARF, RASSF1A and APC1A genes as an independent prognostic factor in colorectal cancer patients. *Int J Oncol* 2013; **42**: 127-133 [PMID: 23128528 DOI: 10.3892/ijo.2012.1682]

105 **Jensen LH**, Rasmussen AA, Byriel L, Kuramochi H, Crüger DG, Lindebjerg J, Danenberg PV, Jakobsen A, Danenberg K. Regulation of MLH1 mRNA and protein expression by promoter methylation in primary colorectal cancer: a descriptive and prognostic cancer marker study. *Cell Oncol* (Dordr) 2013; **36**: 411-419 [PMID: 24027018 DOI: 10.1007/s13402-013-0148-2]

106 **Xing X**, Cai W, Shi H, Wang Y, Li M, Jiao J, Chen M. The prognostic value of CDKN2A hypermethylation in colorectal cancer: a meta-analysis. *Br J Cancer* 2013; **108**: 2542-2548 [PMID: 23703248 DOI: 10.1038/bjc.2013.251]

107 **Katoh H**, Yamashita K, Waraya M, Margalit O, Ooki A, Tamaki H, Sakagami H, Kokubo K, Sidransky D, Watanabe M. Epigenetic silencing of HOPX promotes cancer progression in colorectal cancer. *Neoplasia* 2012; **14**: 559-571 [PMID: 22904674]

108 **Yi JM**, Dhir M, Van Neste L, Downing SR, Jeschke J, Glöckner SC, de Freitas Calmon M, Hooker CM, Funes JM, Boshoff C, Smits KM, van Engeland M, Weijenberg MP, Iacobuzio-Donahue CA, Herman JG, Schuebel KE, Baylin SB, Ahuja N. Genomic and epigenomic integration identifies a prognostic signature in colon cancer. *Clin Cancer Res* 2011; **17**: 1535-1545 [PMID: 21278247]

109 **Baba Y**, Nosho K, Shima K, Huttenhower C, Tanaka N, Hazra A, Giovannucci EL, Fuchs CS, Ogino S. Hypomethylation of the IGF2 DMR in colorectal tumors, detected by bisulfite pyrosequencing, is associated with poor prognosis. *Gastroenterology* 2010; **139**: 1855-1864 [PMID: 20682317 DOI: 10.1053/j.gastro.2010.07.050]

110 **Dallol A**, Al-Maghrabi J, Buhmeida A, Gari MA, Chaudhary AG, Schulten HJ, Abuzenadah AM, Al-Ahwal MS, Sibiany A, Al-Qahtani MH. Methylation of the polycomb group target genes is a possible biomarker for favorable prognosis in colorectal cancer. *Cancer Epidemiol Biomarkers Prev* 2012; **21**: 2069-2075 [PMID: 23010642 DOI: 10.1158/1055-9965.EPI-12-0755]

111 **Yoshimura T**, Nagahara M, Kuo C, Turner RR, Soon-Shiong P, Hoon DS. Lymphovascular invasion of colorectal cancer is correlated to SPARC expression in the tumor stromal microenvironment. *Epigenetics* 2011; **6**: 1001-1011 [PMID: 21725199 DOI: 10.4161/epi.6.8.16063]

112 **Kamimae S**, Yamamoto E, Yamano HO, Nojima M, Suzuki H, Ashida M, Hatahira T, Sato A, Kimura T, Yoshikawa K, Harada T, Hayashi S, Takamaru H, Maruyama R, Kai M, Nishiwaki M, Sugai T, Sasaki Y, Tokino T, Shinomura Y, Imai K, Toyota M. Epigenetic alteration of DNA in mucosal wash fluid predicts invasiveness of colorectal tumors. *Cancer Prev Res* (Phila) 2011; **4**: 674-683 [PMID: 21543345 DOI: 10.1158/1940-6207.CAPR-10-0214]

113 **Takahashi Y**, Iwaya T, Sawada G, Kurashige J, Matsumura T, Uchi R, Ueo H, Takano Y, Eguchi H, Sudo T, Sugimachi K, Yamamoto H, Doki Y, Mori M, Mimori K. Up-regulation of NEK2 by MicroRNA-128 Methylation is Associated with Poor Prognosis in Colorectal Cancer. *Ann Surg Oncol* 2014; **21**: 205-212 [PMID: 24046120]

114 **Zhang Y**, Wang X, Xu B, Wang B, Wang Z, Liang Y, Zhou J, Hu J, Jiang B. Epigenetic silencing of miR-126 contributes to tumor invasion and angiogenesis in colorectal cancer. *Oncol*

Rep 2013; **30**: 1976-1984 [PMID: 23900443 DOI: 10.3892/or.2013.2633]

115 **Ebert MP**, Tänzer M, Balluff B, Burgermeister E, Kretzschmar AK, Hughes DJ, Tetzner R, Lofton-Day C, Rosenberg R, Reinacher-Schick AC, Schulmann K, Tannapfel A, Hofheinz R, Röcken C, Keller G, Langer R, Specht K, Porschen R, Stöhlmacher-Williams J, Schuster T, Ströbel P, Schmid RM. TFAP2E-DKK4 and chemoresistance in colorectal cancer. *N Engl J Med* 2012; **366**: 44-53 [PMID: 22216841 DOI: 10.1056/NEJMoa1009473]

116 **Cheetham S**, Tang MJ, Mesak F, Kennecke H, Owen D, Tai IT. SPARC promoter hypermethylation in colorectal cancers can be reversed by 5-Aza-2′deoxycytidine to increase SPARC expression and improve therapy response. *Br J Cancer* 2008; **98**: 1810-1819 [PMID: 18458674 DOI: 10.1038/sj.bjc.6604377]

117 **Amatu A**, Sartore-Bianchi A, Moutinho C, Belotti A, Bencardino K, Chirico G, Cassingena A, Rusconi F, Esposito A, Nichelatti M, Esteller M, Siena S. Promoter CpG island hypermethylation of the DNA repair enzyme MGMT predicts clinical response to dacarbazine in a phase II study for metastatic colorectal cancer. *Clin Cancer Res* 2013; **19**: 2265-2272 [PMID: 23422094 DOI: 10.1158/1078-0432.CCR-12-3518]

118 **Sawada T**, Yamamoto E, Suzuki H, Nojima M, Maruyama R, Shioi Y, Akasaka R, Kamimae S, Harada T, Ashida M, Kai M, Adachi Y, Yamamoto H, Imai K, Toyota M, Itoh F, Sugai T. Association between genomic alterations and metastatic behavior of colorectal cancer identified by array-based comparative genomic hybridization. *Genes Chromosomes Cancer* 2013; **52**: 140-149 [PMID: 23073979 DOI: 10.1002/gcc.22013]

119 **Spisák S**, Kalmár A, Galamb O, Wichmann B, Sipos F, Péterfia B, Csabai I, Kovalszky I, Semsey S, Tulassay Z, Molnár B. Genome-wide screening of genes regulated by DNA methylation in colon cancer development. *PLoS One* 2012; **7**: e46215 [PMID: 23049694 DOI: 10.1371/journal.pone.0046215]

P- Reviewers: Chu SH, Kuo HC, Nedrebo BS **S- Editor**: Qi Y
L- Editor: A **E- Editor**: Ma S

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.957

World J Gastroenterol 2014 January 28; 20(4): 957-967
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Circulating and stool nucleic acid analysis for colorectal cancer diagnosis

Giulia De Maio, Claudia Rengucci, Wainer Zoli, Daniele Calistri

Giulia De Maio, Claudia Rengucci, Wainer Zoli, Daniele Calistri, Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori IRCCS, Via Piero Maroncelli, 40-47014 Meldola, Italy

Author contributions: Calistri D, De Maio G and Rengucci C conceived the idea for the review and drafted the manuscript; Zoli W revised the paper critically for important intellectual content; all authors approved the final version of the manuscript.

Correspondence to: Daniele Calistri, PhD, Biosciences Laboratory, Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori IRCCS, Via Piero Maroncelli, 40-47014 Meldola, Italy. d.calistri@irst.emr.it

Telephone: +39-0543-739229 Fax: +39-0543-739221

Received: September 26, 2013 Revised: December 5, 2013
Accepted: January 6, 2014
Published online: January 28, 2014

Abstract

In recent years, the need to identify molecular markers characterized by high sensitivity and specificity in detecting and monitoring early and colorectal cancer lesions has increased. Up to now, none of the markers or panels of markers analyzed have met the rigorous standards required of a screening program. The important discovery of circulating nucleic acids in biological fluids has aroused intense scientific interest because of their usefulness in malignant and non malignant diseases. Over time, their yield and stability have been identified and compared with other "standard" biomarkers. The analysis of circulating DNA from blood and stool is a relatively simple and non-invasive procedure, representing a very attractive marker to detect genetic and epigenetic mutations and to monitor disease progression. A correlation between blood and stool biomarkers could also help to enhance currently available diagnostic approaches. However, various processing and analytic problems need to be resolved before such an approach can be applied in clinical practice.

Key words: Colorectal cancer; Circulating DNA; Circulating RNA; Early diagnosis; DNA integrity

Core tip: Although the importance of circulating free DNA is widely recognized, numerous studies evaluating its presence in blood and stool samples have reported analytic variability and non conforming approaches. Nonetheless, circulating free DNA has shown high potential as a biomarker for the early non-invasive detection of cancer and for monitoring disease progression. Population studies are now needed to confirm its usefulness for colorectal cancer diagnosis.

De Maio G, Rengucci C, Zoli W, Calistri D. Circulating and stool nucleic acid analysis for colorectal cancer diagnosis. *World J Gastroenterol* 2014; 20(4): 957-967 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/957.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.957

HISTORY OF CIRCULATING NUCLEIC ACIDS

Circulating nucleic acids in the plasma of healthy and diseased individuals were identified[1] a few years before the discovery of the double helical structure of DNA[2]. Almost 20 years later, circulating DNA was identified in the serum and plasma of subjects with systemic lupus erythematosus[3]. Around the same time, circulating DNA was identified in other diseases characterized by tissue destruction such as hepatitis, metastatic carcinoma and miliary tuberculosis, suggesting that serum DNA might originate from endogenous tissue breakdown[4]. In 1977, levels of circulating DNA in the serum of individuals

with different types of cancer were found to be related to response to radiotherapy treatment. In particular, for the first time, circulating DNA was more accurately quantified using a sensitive radioimmunoassay based on anti-DNA antibodies obtained from lupus erythematosus patients[5]. This quantification revealed that increased or high DNA circulating levels were mainly present in patients characterised by a lack of response to treatment.

The presence of extractable amounts of DNA in the plasma of cancer patients was also identified, suggesting that circulating DNA may be shed from tumours[6]. Other researchers reported findings of *KRAS* and *NRAS* gene mutations from the primary tumor in the plasma and serum of individuals with cancer, providing clear evidence of the origin of circulating DNA from tumors[7-14]. Extracellular nucleic acids, present in different body fluids such as plasma, serum, bronchial lavage, urine and faecal fluids, have aroused the interest of the scientific community in recent years[15,16] representing a valid biomarker for the early, non-invasive detection of cancer or for the monitoring of disease progression. Early diagnosis is fundamental to reduce morbidity and mortality, especially as patients diagnosed at early stages show long-term survival[17].

Unfortunately, the quantity of circulating free DNA in these body fluids is usually low and its isolation remains a challenge. However, rapid technological advances have led to an improved sensitivity and specificity for the detection of cell-free nucleic acids, opening up new possibilities for the non-invasive detection and monitoring of various malignant diseases[15].

ORIGIN OF CIRCULATING FREE DNA

Circulating free DNA is a double-stranded molecule of low molecular weight which, although mainly fragmented in 70-200 base pairs (bp), also has sections up to 21 kilobases in length[18]. In healthy individuals, apoptosis and necrosis of lymphocytes and other nucleated cells are mainly involved in the release of circulating nucleic acids into the blood. Apoptosis leads to DNA degradation in which chromosomal DNA is first cleaved into large fragments (50-300 kb) and then into multiples of nucleosomal units (180-200 bp)[19]. The contents of apoptotic cells are rapidly ingested by phagocytes or neighbouring cells[20] and the DNA is consequently completely digested by DNase Ⅱ in lysosomes[19]. Thus, DNA fragments released by apoptosis may be removed before entering the circulation[19,20]. However, apoptotic DNA is probably the primary source of circulating nucleic acids, especially if we take into account the fact that normal plasma DNA on electrophoresis exhibits band sizes equivalent to whole-number multiples of nucleosomal DNA (185-200 bp)[21]. In cancer patients, the origin of circulating nucleic acids remained unknown for many years. Although increased circulating free DNA levels cannot be regarded as specific to cancer, different size distributions have been observed in cancer patients[22,23]. Currently, the hypothesis on the endogenous origin of circulating DNA proposed by Tan *et al*[3] is widely accepted[4]. Initially, circulating DNA was thought to be a derivative of increased and abnormal apoptotic pathways in cancerous lesions[24,25] because of its ladder pattern revealed by gel electrophoresis similar to the one shown by apoptotic cells[26,27]. However, it must be remembered that apoptosis is a mechanism apparently lost by proliferating cancer cells and that its restoral is highly problematic[9,24,27]. Another hypothesis is that circulating DNA derives from "micrometastatic" tumor cells shed in the circulation. However, some authors reported that the amount of DNA isolated from the plasma of cancer patients was very high and did not correspond to the number of cancer cells present in the circulation[28,29]. Tumor necrosis is thought to be related to high amounts of DNA fragments found in the plasma of patients with large or advanced/metastatic tumors, suggesting that this mechanism may be related to circulating DNA[5,30,31]. However, other pathways could also be involved[4], and probably abnormal DNA degradation or secretion mechanisms may lead to increased DNA levels and differing DNA fragmentation, contributing to the presence of high levels of circulating free DNA[24,32] (Figure 1).

ORIGIN OF CIRCULATING FREE RNA

Less is known on the origin of circulating free RNA[33]. More than 25 years ago, RNA in proteolipid complexes were first identified in the serum of cancer patients[34]. Initially, circulating RNA was found in the serum of healthy individuals and patients with melanoma, breast cancer and hepatocellular carcinoma[35-37]. Numerous studies have reported that specific RNA is present in the plasma of patients with a variety of cancers and that these molecules are more stable than expected[38], suggesting that free circulating RNA is probably protected by vesicles or vesicle-like structures. Apoptosis would also appear to be involved in the release of circulating free RNA, and the binding of proteins or phospholipids may explain the resistance to RNase degradation in the bloodstream[39,40]. Moreover, mRNA and miRNA are found in particles such as exosomes released into the bloodstream, which may help to preserve these nucleic acids in the blood and increase the amount in circulation. In fact, it has been seen that the higher levels of mRNA identified in cancer patients than in healthy individuals are mainly associated with exosome fraction[37].

EARLY DIAGNOSIS OF COLORECTAL CANCER

Colorectal cancer (CRC) incidence and mortality rates vary markedly around the world. However, rates are substantially higher in males than in females[41], representing the third most commonly diagnosed cancer in males and the second in females[42]. CRC is caused by a molecular alteration in the epithelial cells of the colon, specifically,

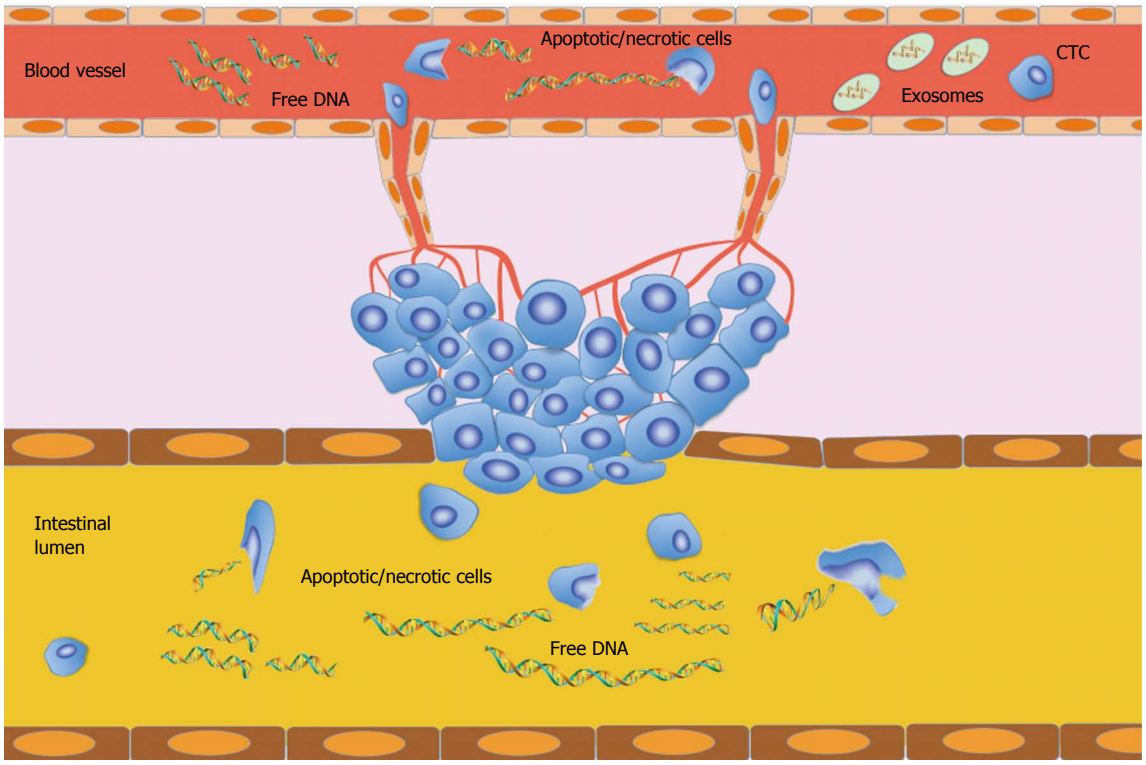


Figure 1 Hypothesis for circulating free DNA development. The primary tumor releases cells into the bloodstream or intestinal lumen. In healthy individuals, apoptosis and necrosis are the main pathways linked to cell degradation and, consequentially, to DNA fragmentation. In cancer patients, in addition to the aforementioned necrosis and apoptosis, there would seem to be abnormal mechanisms of DNA degradation or secretion that increase levels and fragmentation of DNA. CTC: Circulating tumor cells.

in proto-oncogenes and tumor suppressor genes such as *APC*, *KRAS*, *SMAD 2/4* and *p53*. Epigenetic alterations have gained recognition as a key mechanism in colorectal carcinogenesis. In particular, hypermethylation of CpG islands present in gene promoter sequences leads to the inactivation of tumor suppressor genes. The vast majority of tumors (about 50%-80%) present chromosomal instability, while a smaller fraction (10%-15%) is characterized by microsatellite instability (MSI). CRCs with hypermethylation changes in numerous different CpG-rich DNA regions are defined as showing CpG island methylation phenotype (CIMP). CIMP-positive cancer also seems to be associated with MSI and *BRAF* mutations[43,44]. Conversely, hypomethylation of specific sequences may decrease the fidelity of chromosomal segregation[45], suggesting that it could be involved in the chromosomal instability phenotype[46]. DNA methylation changes probably cause adenomatous precursor lesions to progress into malignant tumors.

The importance of screening tools to identify early stage CRC is acknowledged worldwide. Colonoscopy is currently considered to be the "gold standard" for CRC screening. However, it is estimated that less than 60% of eligible individuals over 50 years of age have undergone this test for various reasons, the main one being the invasiveness of the procedure[47]. The immunochemical fecal occult blood test (iFOBT), a non invasive screening CRC approach that uses antibodies against human globulin, has reduced CRC mortality by 15%-33%[48,49]. However, this test is characterized by frequent false-negative and false-positive results, and its sensitivity in detecting precursor lesion such as adenomas is very low (10%-20%)[50].

NUCLEIC ACIDS IN SERUM AND PLASMA

Numerous studies have been published on circulating free DNA in both plasma and serum of different tumor types including colorectal[51-60]. A summary of the most important studies on colorectal cancer patients is shown in Table 1.

It is known that serum contains a higher amount of free circulating DNA than plasma. Different hypotheses for this have been put forward, *e.g.*, an unequal distribution of DNA during separation from whole blood[61]. However, differing levels of circulating free DNA have been observed in experiments using serum and plasma, and the optimal material to process remains open to debate[31].

The most important serum markers for CRC detection are carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9) and tissue inhibitor of matrix metalloproteinases (TIMP)-1. However, CEA is currently the only marker used for prognosis, follow-up and monitoring of disease status. Although high levels of serum CEA are often associated with an increased risk of recurrence and poor prognosis. It is not uncommon to detect normal levels in patients with advanced CRC or early stage patients who subsequently develop recurrence or distant

Table 1 Summary of studies that have tested DNA integrity and genetic alteration markers in blood samples in colorectal cancer

Study	Biomarker(s)	Methods	Case analyzed	Main results	
Flamini *et al*[17], 2006	Serum cfDNA	qRT PCR	CRC: 75	Sensitivity	Specificity
	CEA		HD: 75	81%	73%
	cfDNA and CEA			39%	97%
				88%	71%
Frattini *et al*[51], 2006	Plasma cfDNA	DNA Dipstick Kit	CRC: 70	CRC (T_0) mean value DNA: 495.7 ng/mL	
			HD: 20	CRC (FU at 4 mo) mean value DNA: 170.6 ng/mL	
				CRC (FU at 10 mo) mean value DNA: 240.9 ng/mL	
				CRC (FU at 10 mo)(DFP) mean value DNA: 136.2 ng/mL	
				CRC (FU at 10 mo)(RP) mean value DNA: 694.4 ng/mL	
				HD: mean value DNA 10.3 ng/mL	
				Altered in only 37% of CRC patients	
Umetani *et al*[61], 2006	Plasma cfDNA	ALU qRT PCR	Breast, Colorectal, Thyroid	Mean ± SD value plasma cfDNA: 180 ± 150 pg/μL	
	Serum cfDNA		Cancer: 22	Mean ± SD value serum cfDNA: 970 ± 730 pg/μL	
			Thyroid A: 2		
Umetani *et al*[64], 2006	Absolute serum DNA	ALU qRT PCR	CRC: 32	CRC (Ⅰ-Ⅱ) mean absolute value DNA: 1.63 ng/μL	
	Integrity serum DNA		HD: 51	CRC (Ⅰ-Ⅱ) mean integrity DNA: 0.22 ng/μL	
	Alu repeats (2 sites)			CRC (Ⅲ-Ⅳ) mean absolute DNA: 1.73 ng/μL	
				CRC (Ⅲ-Ⅳ) mean integrity DNA: 0.22 ng/μL	
Boni *et al*[52], 2007	Plasma cfDNA	qRT PCR	CRC: 67	CRC: Mean value DNA 57.93 ng/mL	
	CEA		HD: 67	HD: Mean value DNA 0.85 ng/mL	
				Evaluated in only 47% of patients	
Danese *et al*[65], 2010	Serum cfDNA	RT-PCR	CRC: 118	Sensitivity	Specificity
	CEA		P: 49	83%	92%
			HD: 26	36%	100%
Mead *et al*[31], 2011	Plasma cfDNA	PCR	CRC: 24	AUC: cfDNA: 0.81	
	(4 DNA markers)	ELISA	P: 26	AUC (CEA and cfDNA): 0.855	
	CEA		HD: 35	P: Sensitivity: 83%; Specificity 72%	
Czeiger *et al*[66], 2011	Serum cfDNA	Fluorometric	CRC: 38	Sensitivity	Specificity
		Assay	HD: 34	42%	94%
da Silva Filho *et al*[67], 2013	Serum cfDNA	qPCR	CRC not operated on: 27	CRC not operated on: 0.08-62.10 pg/μL	
	Alu repeats (2 sites)		CRC operated on: 33	CRC operated on: 0.01-186.7 pg/μL	
			HD: 30	HD: 0.01-26.11 pg/μL	
				Mean value range (according to different Alu analysis)	

cfDNA: Cell-free circulating DNA; HD: Healthy donors; A: Adenomas; P: Polyps; T_0: Time 0; FU: Follow up; DFP: Disease free patients; RP: Recurrence patients; AUC: Area under the receiver-operating characteristic (ROC) curve; qRT PCR: Quantitative real time polymerase chain reaction; ELISA: Enzyme-linked immunosorbent assay.

metastases[62]. Thus, the use of CEA assessment for the early diagnosis and monitoring of CRC is limited by relatively poor sensitivity and specificity[63]. In 2006, Frattini *et al*[51] quantified circulating DNA plasma levels of 70 patients submitted to surgery for primary CRC at the time of surgery and during follow-up and comparing this marker with CEA. It was found that circulating DNA levels in all patients at the time of surgery were about 25-fold higher those of 20 healthy donors. Of note, only 37% of patients had altered CEA levels[51]. Moreover, Boni *et al*[52] observed a statistically significant difference in plasma circulating DNA levels between healthy donors and CRC patients. A significant difference was also observed between circulating DNA and CEA values in CRC cases, calculated in only 47% of cases[52].

Sensitivity and specificity of serum free circulating DNA and CEA values were also examined in other studies, confirming high levels of circulating free DNA in CRC patients with respect to healthy individuals. Conversely, sensitivity values of CEA were around 35%, even when specificity was higher[17,65]. Sensitivity and specificity values increased to 88% and 71%, respectively, considering the two markers in combination[17]. Moreover, Frattini *et al*[51] observed that circulating free DNA levels decreased progressively during follow-up in disease-free patients but increased in those who relapsed. This finding is in line with the first observation on circulating free DNA made by Leon *et al*[5] who suggested that the decrease in free DNA in cancer patients before and after treatment may be due to the therapy's inhibitory effect on the proliferation of cancer cells. Conversely, in the early stages of cancer, when little cell death occurs, circulating DNA may already be present in higher than normal concentrations.

Analysing two sites of Alu repeats by a quantitative PCR approach, Umetani *et al*[64] found that serum DNA integrity values were higher in 32 CRC patients than in controls. Interestingly, the serum free circulating DNA concentration was 4- to 6-fold higher in patients than in healthy individuals. This ALU sequencing approach would also appear to be able to discriminate between healthy individuals, CRC patients who have undergone surgery and those who have not been submitted to surgical treatment[67].

These studies revealed a higher sensitivity of circulating DNA then CEA quantitation in CRC patients. Serum

CA19-9, CA72-4 and TIMP-3 are currently being evaluated for screening purposes[68-70], while serum methylation markers such as TAC1, SEPT9 and EYA4 are under investigation as biomarkers for adenoma and early CRC detection[71]. In particular, SEPT9 has been investigated with the aim of improving detection rates of malignant lesion precursors[72], but sensitivity values do not appear to be better than those obtained with stool[73]. Ahlquist *et al*[73] reported higher SEPT9 values than those of the stool DNA multimarker test only for stage Ⅳ CRC. Available data do not, therefore, justify the application of SEPT9 as a single biomarker for the detection of premalignant and early malignant lesions in a screening population. The analysis of paired samples, *i.e.*, stool DNA multimarker test and plasma SEPT 9, showed a sensitivity of 82% and 14% for adenoma detection, respectively, and 87% and 60% for CRC, respectively[73].

Interestingly, a recent meta-analysis by Yang *et al*[74] concluded that stool DNA was not suitable for CRC screening in average- rather than high-risk individuals, yielding a low detection value for precancerous lesions. Amir *et al*[75] demonstrated a correlation between SEPT9_V1 overexpression and drug resistance in various cancer cell lines. The potential of plasma biomarkers to identify individuals at risk of developing drug resistance was also demonstrated by Misale *et al*[76] in a metastatic colorectal cancer population.

Cell free mRNA has also been evaluated in plasma, in particular by the quantification of mRNA levels of hTERT, a ribonucleoprotein involved in the maintenance of correct length of telomeric chromosome ends, overexpressed in a wide number of tumors, including colon and rectal cancers[77,78]. In particular, a correlation has been found between plasma mRNA levels and tumor stage in CRC patients, suggesting that plasma RNA quantification could be useful for early diagnosis and follow up[77,78].

NUCLEIC ACIDS IN STOOL

Non-invasive, no bowel preparation and the sampling design are some of the main advantages of molecular stool analysis. A greater understanding of the molecular pathogenesis and the natural history of CRC has helped researchers to improve methods for molecular stool screening[79]. In 1952, Bader *et al*[80] reported that cancer of the rectum, sigmoid and descending colon could be detected by the cytological analysis of colonocytes. DNA from colonocytes shed in stool can be used to characterize the colonic epithelium involved in carcinogenesis. Colonocytes are, in fact, exfoliated continuously into the fecal stream[81], and their concentration in the intestinal lumen of CRC patients can increase of 4.5-fold with respect to healthy individuals[82]. It has been seen that another important stool element are mucus and its cellular cargo, found on the surface of stool after defecation[83,84]. Colonic mucus would seem to have protective properties that create a niche in which colonocytes are preserved in relative abundance[81,84]. For these reasons, although the mucus present on stool surface after defecation may have been picked up in the distal large bowel, it may also contain cells derived from the entire colorectal mucosa[85]. This would explain why malignant cells from the cecum and other right-sided CRCs can be isolated from stool after defecation[86] despite the luminal contents generally being liquid in the most proximal part of the large bowel. Stool tests based on isolated colonocytes rather than blood markers may offer better results due to the higher rate of neoplastic cell exfoliation, especially when used alone or in combination with current routine diagnostic tests, such as iFOBT[70]. However, as it is extremely difficult to discriminate between normal and malignant cells using standard morphological criteria, considerable interest has arisen in identifying biomarkers secreted by CRC cells rather than normal colonocytes[79]. Adenomas and CRC characteristically exfoliate non-apoptotic colonocytes, unlike normal colonic mucosa, which typically sheds apoptotic colonocytes[87]. The carcinogenetic process can lead to genetic mutations and/or epigenetic alterations that prevent normal colonocytes apoptosis[50]. Non-apoptotic colonocytes shed from diseased mucosa and isolated in the stool can release segments of 200 bp or more in length of intact DNA (L-DNA), making the latter a potentially effective stool biomarker.

A number of authors have proposed different methods to develop valid DNA integrity analysis (DIA) assays to improve sensibility and specificity in the detection of pre-malignant and malignant lesions. Results from studies carried out on stool biomarkers since 2000 are summarized in Table 2.

In one of the first studies to focus on this topic, Boynton *et al*[91] evaluated the length and integrity of L-DNA fragments using oligonucleotide-based hybrid captures with specific target sequences of 200 bp, 400 bp, 800 bp, 1.3 kb, 1.8 kb and 24 kb in PCR reactions; 56% sensitivity and 97% specificity were obtained. The authors concluded that CRC was related to the presence of high molecular weight bands[91]. Another approach to quantify stool L-DNA was carried out using fluorescence primers, capillary electrophoresis and standard curves (fluorescence long DNA, FL-DNA)[92]. In this pilot study on 56 patients and 38 healthy volunteers, FL-DNA evaluation using a cut-off of 25 ng showed a sensitivity of about 76% and a specificity of 93% compared to a specificity of 97% and a sensitivity of only 50% when a non quantitative DNA amplification method was utilized[92].

Long DNA has been also evaluated by analysing human Alu repeats using Real Time PCR assay showing a specificity of 100% but a sensitivity of 44%[96]. To improve accuracy in detecting neoplastic and pre-neoplastic lesions, different combinations of DNA integrity and genetic alteration analyses have been proposed in the last few years. Ahlquist *et al*[88] explored the feasibility of stool assays evaluating panels of selected DNA alterations to discriminate between subjects with colorectal cancer and healthy individuals. The pilot study, using an assay based on L-DNA analysis, *APC*, *TP53* and *KRAS* gene deter-

Table 2 Summary of studies that have tested DNA integrity and genetic alteration markers in stool samples

Study	Biomarker(s)	Methods	Assay for Long DNA	Case analyzed	Sensitivity	Specificity
Ahlquist *et al*[88], 2000	Stool DNA integrity *KRAS, TP53, APC* BAT 26	L-DNA (4 sites) Mutation analysis MSI	Hybrid Capture PCR	CRC: 22 A ⩾ 1 cm: 11 HD: 28	91% 82%	93% NA
Tagore *et al*[89], 2003	Stool DNA integrity *KRAS, TP53, APC* BAT 26	L-DNA (6 sites) Mutation analysis MSI	Hybrid Capture PCR	CRC: 52 AA: 28 HD: 212	64% 57%	96% NA
Calistri *et al*[90], 2003	Stool DNA integrity *KRAS, TP53, APC* D2S123, D5S346, D17S250 BAT 25, BAT 26	L-DNA (8 sites) Mutation analysis MSI	PCR	CRC: 56 HD: 38	62%	97%
Boyton *et al*[91], 2003	Stool DNA integrity	L-DNA (6 sites)	Hybrid Capture PCR	CRC: 27 HD: 77	56%	97%
Calistri *et al*[92], 2004	Stool DNA integrity	FL-DNA (8 sites)	Capillary Electrophoresis	CRC: 85 HD: 59	76%	93%
Whitney *et al*[93], 2004	Stool DNA integrity *KRAS, TP53, APC* BAT 26	L-DNA (4 sites) Mutation analysis MSI	Magnetic Bead-Based Sequence-Specific Purification Capture	CRC: 86 HD: 100	70%	96%
Imperiale *et al*[94], 2004	Stool DNA integrity *KRAS, TP53, APC* BAT 26	L-DNA (4 sites) Mutation analysis MSI	Hybrid Capture PCR	CRC: 31 AA: 407 P: 648 HD: 1423	52% 15% 8%	95% NA NA
Kutzner *et al*[95], 2005	Stool DNA integrity *APC* BAT 26	L-DNA (4 sites) Mutation analysis MSI	Hybrid Capture PCR	CRC: 57 HD: 44	65%	91%
Zou *et al*[96], 2006	Stool DNA integrity	L-DNA: Alu-assay (2 sites)	RT PCR	CRC: 18 HD: 20	44%	100%
Itzkowitz *et al*[97], 2007	Stool DNA integrity *Vimentin*	L-DNA (4 sites Locus D, Locus Y) Methylation analysis	RT PCR	CRC: 40 HD: 122	88%	82%
Abbaszadegan *et al*[98], 2007	Stool DNA integrity p16 BAT 26	L-DNA: (1476 bp fragments) Methylation analysis MSI	PCR	CRC: 25 HD: 20	64%	95%
Ahlquist *et al*[99], 2008	Stool DNA integrity *KRAS, APC, TP53* BAT 26 Stool DNA test 1	L-DNA (4 sites) Mutations Analysis MSI	PCR	CRC: 12 A ⩾ 1 cm: 135 A < 1 cm: 469 P: 341 HD: 1473	25% 17% 4% 5%	96% NA NA NA
Itzkowitz *et al*[47], 2008	Stool DNA integrity *Vimentin*	L-DNA (4 sites, Locus D, Locus Y) Methylation Analysis	RT PCR	CRC: 82 HD: 363	83%	82%
Calistri *et al*[100], 2009	Stool DNA integrity	FL-DNA (8 sites)	Capillary Electrophoresis	CRC: 100 HD: 100	79%	89%
Calistri *et al*[101], 2010	Stool DNA integrity iFOBT	FL-DNA (8 sites) iFOBT	Capillary Electrophoresis	CRC: 26 A HR: 264 A LR: 54 HD: 216	Cancer risk prediction with markers combination	
Kalimutho *et al*[102], 2011	Stool DNA integrity Calprotectin	L-DNA (4 sites) ELISA	QdHPLC	CRC: 28 A: 69 HD: 95	86% 17% 72%	81% NA 75%
Ahlquist *et al*[103], 2012	Stool DNA integrity *KRAS* *NDRG4, BMP3, vimentin, TFP12/α-actine*	L-DNA Mutations Analysis Methylation Analysis	QuARTS	CRC: 252 A ⩾ 1 cm: 133 A < 1 cm: 94 HD: 293	85% 63% 54%	89% NA NA

L-DNA: Long-DNA; FL-DNA: Fluorescence long DNA; CRC: Colorectal cancer; A: Adenoma; AA: Advanced Adenoma; A HR: Adenoma high risk; A LR: Adenoma low risk; HD: Healthy donors; HP: Hyperplasic polyps; NA: Not available; RT-PCR: Real time polymerase chain reaction; QdHPLC: Quantitative denaturing high performance liquid chromatografy; QuARTS: Quantitative allele specific real-time target and signal amplification; MSI: Microsatellite instability.

mination and BAT26 microsatellite instability evaluation, reported 91% sensitivity and 93% specificity in detecting CRC[88]. This high accuracy was not confirmed in a large multicenter study of more than 4000 subjects conducted by Imperiale, who reported obtaining 52% sensitivity in detecting CRC[94]. However, results from Imperiale's study confirmed that molecular analysis identified CRC and adenomas more accurately than the standard FOBT[94]. Another large study performed by Ahlquist some years later using a similar approach based on L-DNA evaluation,

an assessment of 21 tumor-specific point mutations and BAT 26 microsatellite analysis reported a sensitivity of only 25% in detecting cancer[99]. However, the author also showed that sensitivity could be increased to 58% by using a different molecular approach based on 3 broadly informative markers (*KRAS* mutation, *APC* mutator cluster region and *vimentin* gene methylation)[99]. Around the same time, Itzkowitz *et al*[47] used an approach based on the analysis of *vimentin* and different-sized DNA fragments of different loci, obtaining a sensitivity of 83% and a specificity of 82% in detecting CRC. Other combinations of genetic and epigenetic, MSI and DNA integrity markers has been tested over the past ten years in an attempt to define an effective assay for CRC detection. Kalimutho *et al*[102] used an approach based on quantitative-denaturing high performance liquid chromatography detection of *APC, BRAF, KRAS* and *p53* genes to quantify fecal DNA integrity status. Results showed that the four-gene amplification analysis increased sensitivity with respect to single gene amplification, calprotectin evaluation or iFOBT, the latter two assessed using commercial kits[102]. More recently, a next generation stool DNA test based on a quantitative allele-specific real-time target and signal amplification assay was developed with the aim of detecting early-stage CRC, despite the laboriousness and cost of this approach, a sensitivity of 85% for CRC and a specificity of 89% were obtained[103]. Long DNA values have also been evaluated in combination with iFOBT, showing that a combined approach could better predict the presence of tumor or high risk adenoma lesions in the colon[101].

An interesting study by Kanaoka *et al*[104] comparing stool cyclooxygenase-2 (COX-2) and CEA mRNA levels revealed that faecal CEA mRNA specificity was lower than that of COX-2. Furthermore, no significant differences in median faecal CEA mRNA values between CRC patients and control subjects were detected by Koga *et al*[105]. Using the RNA extraction method published by Kanaoka *et al*[104], Hamaya *et al*[106], confirmed significantly higher faecal COX-2 mRNA expression levels in CRC patients than in controls. Moreover, faecal mRNA levels of CEA, E-cad and CD45 showed significantly higher values in CRC patients than in controls.

CONCLUSION

Circulating cell-free nucleic acids are potentially excellent marker for early diagnosis, disease monitoring and more accurate tumour staging.

Molecular markers that could be used to monitor or predict a relapse in a presymptomatic phase of follow-up could have a great impact on the management and, potentially the survival of CRC patients. Several studies have proposed the use of the circulating free DNA quantification as a screening method for CRC diagnosis. Extraction of circulating DNA and RNA from biological fluids *e.g.*, blood and stool, is a simple, relatively noninvasive and low cost procedure, thus representing a very attractive tool to detect genetic and epigenetic mutations, whereas analysis of gene alterations are generally expensive and time consuming. Furthermore, the evaluation of circulating free DNA exclusively derived from tumor cells represents a useful strategy to monitor disease progression. In fact, molecular alterations present in metastatic and primary tumors from the same patient can vary, determining different aggressiveness and/or responsiveness to treatments. Thus, the possibility of monitoring molecular alterations using simple tests based on nucleic acids obtained from blood samples could permit a more efficient assessment of disease status and response to treatments.

Unfortunately, the results from studies carried out in this area also highlight great variability in terms of DNA and RNA concentration, yield, and sensitivity and specificity, indicating the presence of various pre-analytic (serum preparation with or without coagulation accelerator, interval between collection and centrifugation, storage and cryopreservation of samples) and analytic factors (type of extraction with organic solvents, commercial kits, use of magnetic beads) that could influence the diagnostic value of the method. In addition, human faecal RNA is an understudied type of biospecimen due to the difficulty of sample preservation[107]. In particular, sample collection, storage and handling are very important issues for DNA and RNA extracted from stool and could have a substantial impact on the performance of a specific test[107,108]. For these reasons, standardization of sample collection and analysis is fundamental to ensure good reproducibility and large, multicenter studies are needed to clarify the role of these molecular markers in a clinical setting.

ACKNOWLEDGMENTS

The authors would like to thank Ursula Elbling for editing the manuscript.

REFERENCES

1 **Mandel P**, Metais P. [Les acides nucleiques du plasma sanguin chez l'Homme]. *C R Seances Soc Biol Fil* 1948; **142**: 241-243 [PMID: 18875018]
2 **Watson JD**, Crick FH. A structure for deoxyribose nucleic acid. 1953. *Nature* 2003; **421**: 397-398; discussion 396 [PMID: 12569935]
3 **Tan EM**, Schur PH, Carr RI, Kunkel HG. Deoxybonucleic acid (DNA) and antibodies to DNA in the serum of patients with systemic lupus erythematosus. *J Clin Invest* 1966; **45**: 1732-1740 [PMID: 4959277 DOI: 10.1172/JCI105479]
4 **Ziegler A**, Zangemeister-Wittke U, Stahel RA. Circulating DNA: a new diagnostic gold mine? *Cancer Treat Rev* 2002; **28**: 255-271 [PMID: 12435372 DOI: 10.1016/S0305-7372(02)00077-4]
5 **Leon SA**, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res* 1977; **37**: 646-650 [PMID: 837366]
6 **Stroun M**, Anker P, Maurice P, Lyautey J, Lederrey C, Beljanski M. Neoplastic characteristics of the DNA found in the plasma of cancer patients. *Oncology* 1989; **46**: 318-322 [PMID: 2779946]
7 **Sorenson GD**, Pribish DM, Valone FH, Memoli VA, Bzik DJ, Yao SL. Soluble normal and mutated DNA sequences from

single-copy genes in human blood. *Cancer Epidemiol Biomarkers Prev* 1994; **3**: 67-71 [PMID: 8118388]
8 **Vasioukhin V**, Anker P, Maurice P, Lyautey J, Lederrey C, Stroun M. Point mutations of the N-ras gene in the blood plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukaemia. *Br J Haematol* 1994; **86**: 774-779 [PMID: 7918071]
9 **Anker P**, Mulcahy H, Chen XQ, Stroun M. Detection of circulating tumour DNA in the blood (plasma/serum) of cancer patients. *Cancer Metastasis Rev* 1999; **18**: 65-73 [PMID: 10505546]
10 **Sorenson GD**. Detection of mutated KRAS2 sequences as tumor markers in plasma/serum of patients with gastrointestinal cancer. *Clin Cancer Res* 2000; **6**: 2129-2137 [PMID: 10873061]
11 **Kopreski MS**, Benko FA, Borys DJ, Khan A, McGarrity TJ, Gocke CD. Somatic mutation screening: identification of individuals harboring K-ras mutations with the use of plasma DNA. *J Natl Cancer Inst* 2000; **92**: 918-923 [PMID: 10841827 DOI: 10.1093/jnci/92.11.918]
12 **Yamada T**, Nakamori S, Ohzato H, Oshima S, Aoki T, Higaki N, Sugimoto K, Akagi K, Fujiwara Y, Nishisho I, Sakon M, Gotoh M, Monden M. Detection of K-ras gene mutations in plasma DNA of patients with pancreatic adenocarcinoma: correlation with clinicopathological features. *Clin Cancer Res* 1998; **4**: 1527-1532 [PMID: 9626473]
13 **Castells A**, Puig P, Móra J, Boadas J, Boix L, Urgell E, Solé M, Capellà G, Lluís F, Fernández-Cruz L, Navarro S, Farré A. K-ras mutations in DNA extracted from the plasma of patients with pancreatic carcinoma: diagnostic utility and prognostic significance. *J Clin Oncol* 1999; **17**: 578-584 [PMID: 10080602]
14 **Ryan BM**, Lefort F, McManus R, Daly J, Keeling PW, Weir DG, Kelleher D. A prospective study of circulating mutant KRAS2 in the serum of patients with colorectal neoplasia: strong prognostic indicator in postoperative follow up. *Gut* 2003; **52**: 101-108 [PMID: 12477769 DOI: 10.1136/gut.52.1.101]
15 **Tong YK**, Lo YM. Diagnostic developments involving cell-free (circulating) nucleic acids. *Clin Chim Acta* 2006; **363**: 187-196 [PMID: 16126188 DOI: 10.1016/j.cccn.2005.05.048]
16 **Kirsch C**, Weickmann S, Schmidt B, Fleischhacker M. An improved method for the isolation of free-circulating plasma DNA and cell-free DNA from other body fluids. *Ann N Y Acad Sci* 2008; **1137**: 135-139 [PMID: 18837937 DOI: 10.1196/annals.1448.035]
17 **Flamini E**, Mercatali L, Nanni O, Calistri D, Nunziatini R, Zoli W, Rosetti P, Gardini N, Lattuneddu A, Verdecchia GM, Amadori D. Free DNA and carcinoembryonic antigen serum levels: an important combination for diagnosis of colorectal cancer. *Clin Cancer Res* 2006; **12**: 6985-6988 [PMID: 17145818 DOI: 10.1158/1078-0432.CCR-06-1931]
18 **Jahr S**, Hentze H, Englisch S, Hardt D, Fackelmayer FO, Hesch RD, Knippers R. DNA fragments in the blood plasma of cancer patients: quantitations and evidence for their origin from apoptotic and necrotic cells. *Cancer Res* 2001; **61**: 1659-1665 [PMID: 11245480]
19 **Nagata S**, Nagase H, Kawane K, Mukae N, Fukuyama H. Degradation of chromosomal DNA during apoptosis. *Cell Death Differ* 2003; **10**: 108-116 [PMID: 12655299 DOI: 10.1038/sj.cdd.4401161]
20 **Viorritto IC**, Nikolov NP, Siegel RM. Autoimmunity versus tolerance: can dying cells tip the balance? *Clin Immunol* 2007; **122**: 125-134 [PMID: 17029966 DOI: 10.1016/j.clim.2006.07.012]
21 **Giacona MB**, Ruben GC, Iczkowski KA, Roos TB, Porter DM, Sorenson GD. Cell-free DNA in human blood plasma: length measurements in patients with pancreatic cancer and healthy controls. *Pancreas* 1998; **17**: 89-97 [PMID: 9667526]
22 **Wang BG**, Huang HY, Chen YC, Bristow RE, Kassauei K, Cheng CC, Roden R, Sokoll LJ, Chan DW, Shih IeM. Increased plasma DNA integrity in cancer patients. *Cancer Res* 2003; **63**: 3966-3968 [PMID: 12873992]
23 **Ellinger J**, Bastian PJ, Haan KI, Heukamp LC, Buettner R, Fimmers R, Mueller SC, von Ruecker A. Noncancerous PTGS2 DNA fragments of apoptotic origin in sera of prostate cancer patients qualify as diagnostic and prognostic indicators. *Int J Cancer* 2008; **122**: 138-143 [PMID: 17764114 DOI: 10.1002/ijc.23057]
24 **Stroun M**, Lyautey J, Lederrey C, Olson-Sand A, Anker P. About the possible origin and mechanism of circulating DNA apoptosis and active DNA release. *Clin Chim Acta* 2001; **313**: 139-142 [PMID: 11694251]
25 **Tuaeva NO**, Abramova ZI, Sofronov VV. The origin of elevated levels of circulating DNA in blood plasma of premature neonates. *Ann N Y Acad Sci* 2008; **1137**: 27-30 [PMID: 18837920 DOI: 10.1196/annals.1448.043]
26 **Fournié GJ**, Courtin JP, Laval F, Chalé JJ, Pourrat JP, Pujazon MC, Lauque D, Carles P. Plasma DNA as a marker of cancerous cell death. Investigations in patients suffering from lung cancer and in nude mice bearing human tumours. *Cancer Lett* 1995; **91**: 221-227 [PMID: 7767913]
27 **Swarup V**, Rajeswari MR. Circulating (cell-free) nucleic acids--a promising, non-invasive tool for early detection of several human diseases. *FEBS Lett* 2007; **581**: 795-799 [PMID: 17289032 DOI: 10.1016/j.febslet.2007.01.051]
28 **Rhodes CH**, Honsinger C, Porter DM, Sorenson GD. Analysis of the allele-specific PCR method for the detection of neoplastic disease. *Diagn Mol Pathol* 1997; **6**: 49-57 [PMID: 9028737]
29 **Chen X**, Bonnefoi H, Diebold-Berger S, Lyautey J, Lederrey C, Faltin-Traub E, Stroun M, Anker P. Detecting tumor-related alterations in plasma or serum DNA of patients diagnosed with breast cancer. *Clin Cancer Res* 1999; **5**: 2297-2303 [PMID: 10499596]
30 **Nawroz H**, Koch W, Anker P, Stroun M, Sidransky D. Microsatellite alterations in serum DNA of head and neck cancer patients. *Nat Med* 1996; **2**: 1035-1037 [PMID: 8782464]
31 **Mead R**, Duku M, Bhandari P, Cree IA. Circulating tumour markers can define patients with normal colons, benign polyps, and cancers. *Br J Cancer* 2011; **105**: 239-245 [PMID: 21712823]
32 **Drew WL**. Laboratory diagnosis of cytomegalovirus infection and disease in immunocompromised patients. *Curr Opin Infect Dis* 2007; **20**: 408-411 [PMID: 17609601 DOI: 10.1097/QCO.0b013e32821f6010]
33 **González-Masiá JA**, García-Olmo D, García-Olmo DC. Circulating nucleic acids in plasma and serum (CNAPS): applications in oncology. *Onco Targets Ther* 2013; **6**: 819-832 [PMID: 23874104 DOI: 10.2147/OTT.S44668]
34 **Wieczorek AJ**, Sitaramam V, Machleidt W, Rhyner K, Perruchoud AP, Block LH. Diagnostic and prognostic value of RNA-proteolipid in sera of patients with malignant disorders following therapy: first clinical evaluation of a novel tumor marker. *Cancer Res* 1987; **47**: 6407-6412 [PMID: 2445471]
35 **Kopreski MS**, Benko FA, Kwak LW, Gocke CD. Detection of tumor messenger RNA in the serum of patients with malignant melanoma. *Clin Cancer Res* 1999; **5**: 1961-1965 [PMID: 10473072]
36 **Chen XQ**, Bonnefoi H, Pelte MF, Lyautey J, Lederrey C, Movarekhi S, Schaeffer P, Mulcahy HE, Meyer P, Stroun M, Anker P. Telomerase RNA as a detection marker in the serum of breast cancer patients. *Clin Cancer Res* 2000; **6**: 3823-3826 [PMID: 11051224]
37 **Ng EK**, Tsui NB, Lam NY, Chiu RW, Yu SC, Wong SC, Lo ES, Rainer TH, Johnson PJ, Lo YM. Presence of filterable and nonfilterable mRNA in the plasma of cancer patients and healthy individuals. *Clin Chem* 2002; **48**: 1212-1217 [PMID: 12142376]
38 **Tsui NB**, Ng EK, Lo YM. Stability of endogenous and added

RNA in blood specimens, serum, and plasma. *Clin Chem* 2002; **48**: 1647-1653 [PMID: 12324479]
39 **Hasselmann DO**, Rappl G, Tilgen W, Reinhold U. Extracellular tyrosinase mRNA within apoptotic bodies is protected from degradation in human serum. *Clin Chem* 2001; **47**: 1488-1489 [PMID: 11468248]
40 **Kopreski MS**, Gocke CD. Cellular- versus extracellular-based assays. Comparing utility in DNA and RNA molecular marker assessment. *Ann N Y Acad Sci* 2000; **906**: 124-128 [PMID: 10818607 DOI: 10.1111/j.1749-6632.2000.tb06601.x]
41 **Ahnen DJ**, Macrae FA. Colorectal cancer: Epidemiology, risk factors, and protective factors. 2013. Available from: URL: http: //www.uptodate.com/contents/colorectal-cancer-epidemiology-risk-factors-and-protective-factors#H33
42 **Jemal A**, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69-90 [PMID: 21296855]
43 **Weisenberger DJ**, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D, Koh H, Simms L, Barker M, Leggett B, Levine J, Kim M, French AJ, Thibodeau SN, Jass J, Haile R, Laird PW. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet* 2006; **38**: 787-793 [PMID: 16804544 DOI: 10.1038/ng1834]
44 **Hinoue T**, Weisenberger DJ, Pan F, Campan M, Kim M, Young J, Whitehall VL, Leggett BA, Laird PW. Analysis of the association between CIMP and BRAF in colorectal cancer by DNA methylation profiling. *PLoS One* 2009; **4**: e8357 [PMID: 20027224]
45 **Ji W**, Hernandez R, Zhang XY, Qu GZ, Frady A, Varela M, Ehrlich M. DNA demethylation and pericentromeric rearrangements of chromosome 1. *Mutat Res* 1997; **379**: 33-41 [PMID: 9330620 DOI: 10.1016/j.bbr.2011.03.031]
46 **Goel A**, Nagasaka T, Arnold CN, Inoue T, Hamilton C, Niedzwiecki D, Compton C, Mayer RJ, Goldberg R, Bertagnolli MM, Boland CR. The CpG island methylator phenotype and chromosomal instability are inversely correlated in sporadic colorectal cancer. *Gastroenterology* 2007; **132**: 127-138 [PMID: 17087942 DOI: 10.1053/j.gastro.2006.09.018]
47 **Itzkowitz S**, Brand R, Jandorf L, Durkee K, Millholland J, Rabeneck L, Schroy PC, Sontag S, Johnson D, Markowitz S, Paszat L, Berger BM. A simplified, noninvasive stool DNA test for colorectal cancer detection. *Am J Gastroenterol* 2008; **103**: 2862-2870 [PMID: 18759824]
48 **Brenner DE**, Rennert G. Fecal DNA biomarkers for the detection of colorectal neoplasia: attractive, but is it feasible? *J Natl Cancer Inst* 2005; **97**: 1107-1109 [PMID: 16077063 DOI: 10.1093/jnci/dji244]
49 **Lenhard K**, Bommer GT, Asutay S, Schauer R, Brabletz T, Göke B, Lamerz R, Kolligs FT. Analysis of promoter methylation in stool: a novel method for the detection of colorectal cancer. *Clin Gastroenterol Hepatol* 2005; **3**: 142-149 [PMID: 15704048]
50 **Kanthan R**, Senger JL, Kanthan SC. Fecal molecular markers for colorectal cancer screening. *Gastroenterol Res Pract* 2012; **2012**: 184343 [PMID: 22969796 DOI: 10.1155/2012/184343]
51 **Frattini M**, Gallino G, Signoroni S, Balestra D, Battaglia L, Sozzi G, Leo E, Pilotti S, Pierotti MA. Quantitative analysis of plasma DNA in colorectal cancer patients: a novel prognostic tool. *Ann N Y Acad Sci* 2006; **1075**: 185-190 [PMID: 17108210 DOI: 10.1196/annals.1368.025]
52 **Boni L**, Cassinotti E, Canziani M, Dionigi G, Rovera F, Dionigi R. Free circulating DNA as possible tumour marker in colorectal cancer. *Surg Oncol* 2007; **16** Suppl 1: S29-S31 [PMID: 18024018 DOI: 10.1016/]
53 **Tomita H**, Ichikawa D, Sai S, Morimura R, Murayama Y, Komatsu S, Ikoma D, Tani N, Ikoma H, Fujiwara H, Kikuchi S, Okamoto K, Ochiai T, Kokuba Y, Sakakura C, Sonoyama T, Hagiwara A, Otsuji E. [Quantification of circulating plasma DNA fragments as tumor markers in patients with esophageal and gastric cancer]. *Gan To Kagaku Ryoho* 2007; **34**: 1908-1910 [PMID: 18219849 DOI: 10.1016/j.suronc]
54 **Kolesnikova EV**, Tamkovich SN, Bryzgunova OE, Shelestyuk PI, Permyakova VI, Vlassov VV, Tuzikov AS, Laktionov PP, Rykova EY. Circulating DNA in the blood of gastric cancer patients. *Ann N Y Acad Sci* 2008; **1137**: 226-231 [PMID: 18837952 DOI: 10.1196/annals.1448.009]
55 **Catarino R**, Ferreira MM, Rodrigues H, Coelho A, Nogal A, Sousa A, Medeiros R. Quantification of free circulating tumor DNA as a diagnostic marker for breast cancer. *DNA Cell Biol* 2008; **27**: 415-421 [PMID: 18694299 DOI: 10.1089/dna.2008.0744]
56 **Yoon KA**, Park S, Lee SH, Kim JH, Lee JS. Comparison of circulating plasma DNA levels between lung cancer patients and healthy controls. *J Mol Diagn* 2009; **11**: 182-185 [PMID: 19324991]
57 **Ellinger J**, Müller SC, Stadler TC, Jung A, von Ruecker A, Bastian PJ. The role of cell-free circulating DNA in the diagnosis and prognosis of prostate cancer. *Urol Oncol* 2011; **29**: 124-129 [PMID: 19762255]
58 **Dobrzycka B**, Terlikowski SJ, Mazurek A, Kowalczuk O, Niklinska W, Chyczewski L, Kulikowski M. Circulating free DNA, p53 antibody and mutations of KRAS gene in endometrial cancer. *Int J Cancer* 2010; **127**: 612-621 [PMID: 19960433 DOI: 10.1002/ijc.25077]
59 **Liggett T**, Melnikov A, Yi QL, Replogle C, Brand R, Kaul K, Talamonti M, Abrams RA, Levenson V. Differential methylation of cell-free circulating DNA among patients with pancreatic cancer versus chronic pancreatitis. *Cancer* 2010; **116**: 1674-1680 [PMID: 20143430 DOI: 10.1002/cncr.24893]
60 **van der Drift MA**, Hol BE, Klaassen CH, Prinsen CF, van Aarssen YA, Donders R, van der Stappen JW, Dekhuijzen PN, van der Heijden HF, Thunnissen FB. Circulating DNA is a non-invasive prognostic factor for survival in non-small cell lung cancer. *Lung Cancer* 2010; **68**: 283-287 [PMID: 19632736 DOI: 10.1016/j.lungcan.2009.06.021]
61 **Umetani N**, Hiramatsu S, Hoon DS. Higher amount of free circulating DNA in serum than in plasma is not mainly caused by contaminated extraneous DNA during separation. *Ann N Y Acad Sci* 2006; **1075**: 299-307 [PMID: 17108224 DOI: 10.1196/annals.1368.040]
62 **Hara M**, Kanemitsu Y, Hirai T, Komori K, Kato T. Negative serum carcinoembryonic antigen has insufficient accuracy for excluding recurrence from patients with Dukes C colorectal cancer: analysis with likelihood ratio and posttest probability in a follow-up study. *Dis Colon Rectum* 2008; **51**: 1675-1680 [PMID: 18633674 DOI: 10.1007/s10350-008-9406-1]
63 **Winawer S**, Fletcher R, Rex D, Bond J, Burt R, Ferrucci J, Ganiats T, Levin T, Woolf S, Johnson D, Kirk L, Litin S, Simmang C. Colorectal cancer screening and surveillance: clinical guidelines and rationale-Update based on new evidence. *Gastroenterology* 2003; **124**: 544-560 [PMID: 12557158]
64 **Umetani N**, Kim J, Hiramatsu S, Reber HA, Hines OJ, Bilchik AJ, Hoon DS. Increased integrity of free circulating DNA in sera of patients with colorectal or periampullary cancer: direct quantitative PCR for ALU repeats. *Clin Chem* 2006; **52**: 1062-1069 [PMID: 16723681]
65 **Danese E**, Montagnana M, Minicozzi AM, De Matteis G, Scudo G, Salvagno GL, Cordiano C, Lippi G, Guidi GC. Real-time polymerase chain reaction quantification of free DNA in serum of patients with polyps and colorectal cancers. *Clin Chem Lab Med* 2010; **48**: 1665-1668 [PMID: 20704532 DOI: 10.1515/CCLM.2010.301]
66 **Czeiger D**, Shaked G, Eini H, Vered I, Belochitski O, Avriel A, Ariad S, Douvdevani A. Measurement of circulating cell-free DNA levels by a new simple fluorescent test in patients with primary colorectal cancer. *Am J Clin Pathol* 2011; **135**: 264-270 [PMID: 21228367]
67 **da Silva Filho BF**, Gurgel AP, Neto MÁ, de Azevedo DA,

de Freitas AC, Silva Neto Jda C, Silva LA. Circulating cell-free DNA in serum as a biomarker of colorectal cancer. *J Clin Pathol* 2013; **66**: 775-778 [PMID: 23833048 DOI: 10.1136/jclinpath-2013-201521]

68 **Holten-Andersen MN**, Christensen IJ, Nielsen HJ, Stephens RW, Jensen V, Nielsen OH, Sørensen S, Overgaard J, Lilja H, Harris A, Murphy G, Brünner N. Total levels of tissue inhibitor of metalloproteinases 1 in plasma yield high diagnostic sensitivity and specificity in patients with colon cancer. *Clin Cancer Res* 2002; **8**: 156-164 [PMID: 11801553]

69 **Carpelan-Holmström M**, Louhimo J, Stenman UH, Alfthan H, Haglund C. CEA, CA 19-9 and CA 72-4 improve the diagnostic accuracy in gastrointestinal cancers. *Anticancer Res* 2002; **22**: 2311-2316 [PMID: 12174919]

70 **Carpelan-Holmström M**, Louhimo J, Stenman UH, Alfthan H, Järvinen H, Haglund C. CEA, CA 242, CA 19-9, CA 72-4 and hCGbeta in the diagnosis of recurrent colorectal cancer. *Tumour Biol* 2004; **25**: 228-234 [PMID: 15627885]

71 **Liu Y**, Tham CK, Ong SY, Ho KS, Lim JF, Chew MH, Lim CK, Zhao Y, Tang CL, Eu KW. Serum methylation levels of TAC1. SEPT9 and EYA4 as diagnostic markers for early colorectal cancers: a pilot study. *Biomarkers* 2013; **18**: 399-405 [PMID: 23862763]

72 **Grützmann R**, Molnar B, Pilarsky C, Habermann JK, Schlag PM, Saeger HD, Miehlke S, Stolz T, Model F, Roblick UJ, Bruch HP, Koch R, Liebenberg V, Devos T, Song X, Day RH, Sledziewski AZ, Lofton-Day C. Sensitive detection of colorectal cancer in peripheral blood by septin 9 DNA methylation assay. *PLoS One* 2008; **3**: e3759 [PMID: 19018278 DOI: 10.1371/journal.pone.0003759]

73 **Ahlquist DA**, Taylor WR, Mahoney DW, Zou H, Domanico M, Thibodeau SN, Boardman LA, Berger BM, Lidgard GP. The stool DNA test is more accurate than the plasma septin 9 test in detecting colorectal neoplasia. *Clin Gastroenterol Hepatol* 2012; **10**: 272-7.e1 [PMID: 22019796 DOI: 10.1016/j.cgh.2011.10.008]

74 **Yang H**, Xia BQ, Jiang B, Wang G, Yang YP, Chen H, Li BS, Xu AG, Huang YB, Wang XY. Diagnostic value of stool DNA testing for multiple markers of colorectal cancer and advanced adenoma: a meta-analysis. *Can J Gastroenterol* 2013; **27**: 467-475 [PMID: 23936877]

75 **Amir S**, Mabjeesh NJ. SEPT9_V1 protein expression is associated with human cancer cell resistance to microtubule-disrupting agents. *Cancer Biol Ther* 2007; **6**: 1926-1931 [PMID: 18075300]

76 **Misale S**, Yaeger R, Hobor S, Scala E, Janakiraman M, Liska D, Valtorta E, Schiavo R, Buscarino M, Siravegna G, Bencardino K, Cercek A, Chen CT, Veronese S, Zanon C, Sartore-Bianchi A, Gambacorta M, Gallicchio M, Vakiani E, Boscaro V, Medico E, Weiser M, Siena S, Di Nicolantonio F, Solit D, Bardelli A. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. *Nature* 2012; **486**: 532-536 [PMID: 22722830 DOI: 10.1038/nature11156]

77 **Terrin L**, Rampazzo E, Pucciarelli S, Agostini M, Bertorelle R, Esposito G, DelBianco P, Nitti D, De Rossi A. Relationship between tumor and plasma levels of hTERT mRNA in patients with colorectal cancer: implications for monitoring of neoplastic disease. *Clin Cancer Res* 2008; **14**: 7444-7451 [PMID: 19010861 DOI: 10.1158/1078-0432.CCR-08-0478]

78 **Pucciarelli S**, Rampazzo E, Briarava M, Maretto I, Agostini M, Digito M, Keppel S, Friso ML, Lonardi S, De Paoli A, Mescoli C, Nitti D, De Rossi A. Telomere-specific reverse transcriptase (hTERT) and cell-free RNA in plasma as predictors of pathologic tumor response in rectal cancer patients receiving neoadjuvant chemoradiotherapy. *Ann Surg Oncol* 2012; **19**: 3089-3096 [PMID: 22395986 DOI: 10.1245/s10434-012-2272-z]

79 **Davies RJ**, Miller R, Coleman N. Colorectal cancer screening: prospects for molecular stool analysis. *Nat Rev Cancer* 2005; **5**: 199-209 [PMID: 15738983 DOI: 10.1038/nrc1545]

80 **Bader GM**, Papanicolaou GN. The application of cytology in the diagnosis of cancer of the rectum, sigmoid, and descending colon. *Cancer* 1952; **5**: 307-314 [PMID: 14905415]

81 **Ahlquist DA**, Harrington JJ, Burgart LJ, Roche PC. Morphometric analysis of the "mucocellular layer" overlying colorectal cancer and normal mucosa: relevance to exfoliation and stool screening. *Hum Pathol* 2000; **31**: 51-57 [PMID: 10665913]

82 **Loktionov A**, O'Neill IK, Silvester KR, Cummings JH, Middleton SJ, Miller R. Quantitation of DNA from exfoliated colonocytes isolated from human stool surface as a novel noninvasive screening test for colorectal cancer. *Clin Cancer Res* 1998; **4**: 337-342 [PMID: 9516920]

83 **Baig MK**, Stebbing JF, Marks CG. Anal canal metastases from left sided colorectal cancer. *Colorectal Dis* 2002; **4**: 371-372 [PMID: 12780585 DOI: 10.1046/j.1463-1318.2002.00405.x]

84 **Loktionov A**. Cell exfoliation in the human colon: myth, reality and implications for colorectal cancer screening. *Int J Cancer* 2007; **120**: 2281-2289 [PMID: 17351899 DOI: 10.1002/ijc.22647]

85 **White V**, Scarpini C, Barbosa-Morais NL, Ikelle E, Carter S, Laskey RA, Miller R, Coleman N. Isolation of stool-derived mucus provides a high yield of colonocytes suitable for early detection of colorectal carcinoma. *Cancer Epidemiol Biomarkers Prev* 2009; **18**: 2006-2013 [PMID: 19589912 DOI: 10.1158/1055-9965.EPI-08-1145]

86 **Davies RJ**, Freeman A, Morris LS, Bingham S, Dilworth S, Scott I, Laskey RA, Miller R, Coleman N. Analysis of minichromosome maintenance proteins as a novel method for detection of colorectal cancer in stool. *Lancet* 2002; **359**: 1917-1919 [PMID: 12057556 DOI: 10.1016/S0140-6736(02)08739-1]

87 **Bedi A**, Pasricha PJ, Akhtar AJ, Barber JP, Bedi GC, Giardiello FM, Zehnbauer BA, Hamilton SR, Jones RJ. Inhibition of apoptosis during development of colorectal cancer. *Cancer Res* 1995; **55**: 1811-1816 [PMID: 7728743]

88 **Ahlquist DA**, Skoletsky JE, Boynton KA, Harrington JJ, Mahoney DW, Pierceall WE, Thibodeau SN, Shuber AP. Colorectal cancer screening by detection of altered human DNA in stool: feasibility of a multitarget assay panel. *Gastroenterology* 2000; **119**: 1219-1227 [PMID: 11054379]

89 **Tagore KS**, Lawson MJ, Yucaitis JA, Gage R, Orr T, Shuber AP, Ross ME. Sensitivity and specificity of a stool DNA multitarget assay panel for the detection of advanced colorectal neoplasia. *Clin Colorectal Cancer* 2003; **3**: 47-53 [PMID: 12777192 DOI: 10.3816/CCC.2003.n.011]

90 **Calistri D**, Rengucci C, Bocchini R, Saragoni L, Zoli W, Amadori D. Fecal multiple molecular tests to detect colorectal cancer in stool. *Clin Gastroenterol Hepatol* 2003; **1**: 377-383 [PMID: 15017656]

91 **Boynton KA**, Summerhayes IC, Ahlquist DA, Shuber AP. DNA integrity as a potential marker for stool-based detection of colorectal cancer. *Clin Chem* 2003; **49**: 1058-1065 [PMID: 12816901 DOI: 10.1373/49.7.1058]

92 **Calistri D**, Rengucci C, Lattuneddu A, Francioni G, Polifemo AM, Nanni O, Saragoni L, Monti F, Ravaioli A, Zoli W, Amadori D. Detection of colorectal cancer by a quantitative fluorescence determination of DNA amplification in stool. *Neoplasia* 2004; **6**: 536-540 [PMID: 15548362 DOI: 10.1593/neo.04190]

93 **Whitney D**, Skoletsky J, Moore K, Boynton K, Kann L, Brand R, Syngal S, Lawson M, Shuber A. Enhanced retrieval of DNA from human fecal samples results in improved performance of colorectal cancer screening test. *J Mol Diagn* 2004; **6**: 386-395 [PMID: 15507679 DOI: 10.1016/S1525-1578(10)60536-3]

94 **Imperiale TF**, Ransohoff DF, Itzkowitz SH, Turnbull BA, Ross ME. Fecal DNA versus fecal occult blood for colorectal-cancer screening in an average-risk population. *N Engl J Med*

2004; **351**: 2704-2714 [PMID: 15616205 DOI: 10.1056/NEJMoa033403]
95 **Kutzner N**, Hoffmann I, Linke C, Thienel T, Grzegorczyk M, Urfer W, Martin D, Winde G, Traska T, Hohlbach G, Müller KM, Kuhnen C, Müller O. Non-invasive detection of colorectal tumours by the combined application of molecular diagnosis and the faecal occult blood test. *Cancer Lett* 2005; **229**: 33-41 [PMID: 16157216]
96 **Zou H**, Harrington JJ, Klatt KK, Ahlquist DA. A sensitive method to quantify human long DNA in stool: relevance to colorectal cancer screening. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 1115-1119 [PMID: 16775168 DOI: 10.1158/1055-9965.EPI-05-0992]
97 **Itzkowitz SH**, Jandorf L, Brand R, Rabeneck L, Schroy PC, Sontag S, Johnson D, Skoletsky J, Durkee K, Markowitz S, Shuber A. Improved fecal DNA test for colorectal cancer screening. *Clin Gastroenterol Hepatol* 2007; **5**: 111-117 [PMID: 17161655 DOI: 10.1016/j.cgh.2006.10.006]
98 **Abbaszadegan MR**, Tavasoli A, Velayati A, Sima HR, Vosooghinia H, Farzadnia M, Asadzedeh H, Gholamin M, Dadkhah E, Aarabi A. Stool-based DNA testing, a new noninvasive method for colorectal cancer screening, the first report from Iran. *World J Gastroenterol* 2007; **13**: 1528-1533 [PMID: 17461444]
99 **Ahlquist DA**, Sargent DJ, Loprinzi CL, Levin TR, Rex DK, Ahnen DJ, Knigge K, Lance MP, Burgart LJ, Hamilton SR, Allison JE, Lawson MJ, Devens ME, Harrington JJ, Hillman SL. Stool DNA and occult blood testing for screen detection of colorectal neoplasia. *Ann Intern Med* 2008; **149**: 441-50, W81 [PMID: 18838724 DOI: 10.7326/0003-4819-149-7-200810070-00004]
100 **Calistri D**, Rengucci C, Molinari C, Ricci E, Cavargini E, Scarpi E, Milandri GL, Fabbri C, Ravaioli A, Russo A, Amadori D, Silvestrini R. Quantitative fluorescence determination of long-fragment DNA in stool as a marker for the early detection of colorectal cancer. *Cell Oncol* 2009; **31**: 11-17 [PMID: 19096146]
101 **Calistri D**, Rengucci C, Casadei Gardini A, Frassineti GL, Scarpi E, Zoli W, Falcini F, Silvestrini R, Amadori D. Fecal DNA for noninvasive diagnosis of colorectal cancer in immunochemical fecal occult blood test-positive individuals. *Cancer Epidemiol Biomarkers Prev* 2010; **19**: 2647-2654 [PMID: 20929882 DOI: 10.1158/1055-9965.EPI-10-0291]
102 **Kalimutho M**, Del Vecchio Blanco G, Cretella M, Mannisi E, Sileri P, Formosa A, Pallone F, Federici G, Bernardini S. A simplified, non-invasive fecal-based DNA integrity assay and iFOBT for colorectal cancer detection. *Int J Colorectal Dis* 2011; **26**: 583-592 [PMID: 21225430 DOI: 10.1007/s00384-010-112]
103 **Ahlquist DA**, Zou H, Domanico M, Mahoney DW, Yab TC, Taylor WR, Butz ML, Thibodeau SN, Rabeneck L, Paszat LF, Kinzler KW, Vogelstein B, Bjerregaard NC, Laurberg S, Sørensen HT, Berger BM, Lidgard GP. Next-generation stool DNA test accurately detects colorectal cancer and large adenomas. *Gastroenterology* 2012; **142**: 248-56; quiz e25-6 [PMID: 22062357 DOI: 10.1053/j.gastro.2011.10.031]
104 **Kanaoka S**, Yoshida K, Miura N, Sugimura H, Kajimura M. Potential usefulness of detecting cyclooxygenase 2 messenger RNA in feces for colorectal cancer screening. *Gastroenterology* 2004; **127**: 422-427 [PMID: 15300574 DOI: 10.1053/j.gastro.2004.05.022]
105 **Koga Y**, Yasunaga M, Moriya Y, Akasu T, Fujita S, Yamamoto S, Kozu T, Baba H, Matsumura Y. Detection of colorectal cancer cells from feces using quantitative real-time RT-PCR for colorectal cancer diagnosis. *Cancer Sci* 2008; **99**: 1977-1983 [PMID: 19016757 DOI: 10.1111/j.1349-7006.2008.00954]
106 **Hamaya Y**, Yoshida K, Takai T, Ikuma M, Hishida A, Kanaoka S. Factors that contribute to faecal cyclooxygenase-2 mRNA expression in subjects with colorectal cancer. *Br J Cancer* 2010; **102**: 916-921 [PMID: 20145612 DOI: 10.1038/sj.bjc.6605564]
107 **Yu YJ**, Majumdar AP, Nechvatal JM, Ram JL, Basson MD, Heilbrun LK, Kato I. Exfoliated cells in stool: a source for reverse transcription-PCR-based analysis of biomarkers of gastrointestinal cancer. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 455-458 [PMID: 18268130 DOI: 10.1158/1055-9965]
108 **Olson J**, Whitney DH, Durkee K, Shuber AP. DNA stabilization is critical for maximizing performance of fecal DNA-based colorectal cancer tests. *Diagn Mol Pathol* 2005; **14**: 183-191 [PMID: 16106201]

P- Reviewers: Aglietta M, Beaulieu JF, Pavlidis TE
S- Editor: Gou SX **L- Editor**: A **E- Editor**: Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.968

World J Gastroenterol 2014 January 28; 20(4): 968-977
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Cysteinyl leukotrienes and their receptors: Bridging inflammation and colorectal cancer

Sayeh Savari, Katyayni Vinnakota, Yuan Zhang, Anita Sjölander

Sayeh Savari, Katyayni Vinnakota, Yuan Zhang, Anita Sjölander, Division of Cell and Experimental Pathology, Department of Laboratory Medicine, Lund University, Skåne University Hospital, SE-205 02 Malmö, Sweden

Author contributions: Savari S and Vinnakota K contributed equally to this work; all authors discussed, read and approved the manuscript.

Correspondence to: Anita Sjölander, Professor, Division of Cell and Experimental Pathology, Department of Laboratory Medicine, Lund University, Skåne University Hospital, Jan Waldenströms gata 35, SE-205 02 Malmö, Sweden. anita.sjolander@med.lu.se

Telephone: +46-40-391168 Fax: +46-40-391177

Received: October 6, 2013 Revised: November 16, 2013
Accepted: December 5, 2013
Published online: January 28, 2014

Abstract

Long-standing inflammation has emerged as a hallmark of neoplastic transformation of epithelial cells and may be a limiting factor of successful conventional tumor therapies. A complex milieu composed of distinct stromal and immune cells, soluble factors and inflammatory mediators plays a crucial role in supporting and promoting various types of cancers. An augmented inflammatory response can predispose a patient to colorectal cancer (CRC). Common risk factors associated with CRC development include diet and lifestyle, altered intestinal microbiota and commensals, and chronic inflammatory bowel diseases. Cysteinyl leukotrienes are potent inflammatory metabolites synthesized from arachidonic acid and have a broad range of functions involved in the etiology of various pathologies. This review discusses the important role of cysteinyl leukotriene signaling in linking inflammation and CRC.

Key words: Eicosanoids; Cysteinyl leukotrienes; CysLT$_1$R; CysLT$_2$R; Inflammation; Colorectal cancer

Core tip: Despite several advances in diagnostic and therapeutic options, colorectal cancer (CRC) continues to be a major health problem and one of the leading causes of cancer-related deaths. The inflammatory milieu has been widely recognized as one of the enabling characteristics of cancer development. Cysteinyl leukotrienes are pro-inflammatory eicosanoids implicated in chronic inflammatory bowel diseases and CRC development. Hence, targeting cysteinyl leukotrienes and their receptors could provide alternative therapeutic approaches or be used in combination with existing therapies for more efficient treatment of CRC.

Savari S, Vinnakota K, Zhang Y, Sjölander A. Cysteinyl leukotrienes and their receptors: Bridging inflammation and colorectal cancer. *World J Gastroenterol* 2014; 20(4): 968-977 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/968.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.968

INTRODUCTION

Colorectal cancer (CRC) is a global health care burden, with more than 1 million new cases diagnosed every year. It is the third most common malignancy and the fourth leading cause of cancer-related deaths worldwide[1]. A diet high in fat but low in fiber, excessive alcohol consumption, obesity and lack of physical activity, disruption of normal gut microbiota and the presence of long-term inflammatory bowel diseases (IBDs) such as ulcerative colitis (UC) and Crohn's disease (CD) predispose to CRC. Inflammation is a host-driven response to internal and external stimuli to counter non-self or self-molecules and maintain tissue homeostasis. However, chronic inflamma-

tion can be a major health problem in allergic, cardiovascular, fibrotic, local and systemic inflammatory diseases and several cancers[2-9]. In 1863, Rudolf Virchow was the first to speculate about the role of long-term inflammation in cancer based on his observations that cancerous tissues were frequently infiltrated by leukocytes[10]. Current epidemiological data indicate that more than 25% of all cancers are related to long-term infections and other types of unresolved inflammation[11-13]. Evidence from observational studies and randomized trials concerning the protective action of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin have indicated not only a reduced long-term risk of esophageal, gastric, biliary, breast, prostate, lung, and CRC but also a lowered risk of metastasis[14-18]. Inflammation present in the tumor microenvironment is characterized by high leukocyte infiltration, ranging in size, distribution and composition, such as tumor-associated macrophages (TAMs), mast cells, dendritic cells (DCs), natural killer cells (NKs), neutrophils, eosinophils and lymphocytes[19-21]. These cells produce a variety of cytotoxic mediators such as reactive oxygen and nitrogen species, serine and cysteine proteases, membrane-perforating agents, matrix metalloproteinases, pro-inflammatory cytokines, interferons (IFNs) and increased levels of enzymes such as cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX) and phospholipase A_2 (PLA_2), hence contributing to carcinogenesis[22-25]. This review addresses the role of cysteinyl leukotrienes in inflammation-induced colorectal carcinogenesis.

EICOSANOIDS

Eicosanoids, from the Greek word "eicosa" meaning "20," are biologically active lipophilic molecules predominantly metabolized from arachidonic acid (AA), a 20-carbon polyunsaturated essential fatty acid, that are involved in physiological processes such as inflammation[14]. AA belongs to the ω-6 family of polyunsaturated fatty acids and is usually found esterified at the second carbon position in the phospholipids of membranes. It serves as a precursor to several lipid pro-inflammatory mediators such as prostaglandins (PGs), prostacyclins, thromboxanes (TXAs), lipoxins and leukotrienes (LTs), which have individual as well as overlapping functions in acute and chronic inflammation[26]. Aberrant AA metabolism is often linked to production of pro-inflammatory eicosanoids, chronic inflammatory diseases and carcinogenesis. The first eicosanoids were discovered in the 1960s, although in 1930 scientists had found that certain substances present in biological fluids such as sputum had the potential to induce contraction and relaxation in smooth muscles; they termed them"slow-reacting substances of anaphylaxis." Despite these early observations, it was not until 1979 that "leukotrienes" were identified and defined by Samuelsson and co-workers for their biological effects in inflammatory processes[27]. On account of their fundamental and seminal work on different eicosanoids, mainly the prostaglandins, and for their discovery of the role of anti-inflammatory compounds such as aspirin on prostaglandin metabolism, the scientists Bengt Samuelsson, John Vane and Sune Bergström were awarded the Nobel Prize for Physiology and Medicine in 1982.

AA, which is stored as diacylglycerol (DAG), is released from the phosphatidylinositol 4,5-bisphosphate (PIP_2) present in the outer nuclear envelope of cells, from which it is mobilized into the cytoplasm either by activation of calcium-dependent cytosolic PLA_2 or by the combined action of phospholipase C (PLC) and DAG lipase[26,28]. Once in the cytosol, AA can be enzymatically metabolized in a three-directional manner either by cytochrome P450 or by the COX pathway into prostaglandins, prostacyclins or thromboxanes, or through the 5-LOX pathway into leukotrienes A_4 (LTA_4), B_4 (LTB_4), C_4 (LTC_4), D_4 (LTD_4) and E_4 (LTE_4) (Figure 1). The last three alternative derivatives to LTA_4 are collectively termed "cysteinyl leukotrienes" (CysLTs) owing to the presence of a cysteine residue and are structurally similar but functionally distinct. The role of these eicosanoids in maintaining intestinal epithelial cell homeostasis is well documented[29-32]. Various epidemiological, clinical, and laboratory studies have shown that dysregulation of the COX and LOX pathways results in chronic inflammation and subsequently cancer[14,29].

LEUKOTRIENES AND THEIR RECEPTORS

The term leukotriene is derived from the two words *leuko* for white blood cells and *trienes*, meaning three conjugated double bonds, indicating that the ability to generate LTs from AA is largely restricted to leukocytes[28].

Synthesis of LTs is initiated by 5-LOX in concert with 5-lipoxygenase-activating protein (FLAP). The latter does not exhibit any enzymatic activity but facilitates the interaction between 5-LOX and its substrate AA. The first step in this pathway is oxygenation of AA to yield unstable 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which immediately undergoes dehydration to form LTA_4. Further metabolism of LTA_4 either generates LTB_4 by the action of LTA_4 hydrolase (LTA_4-H) or leads to conjugation with glutathione in the presence of LTC_4 synthase (LTC_4-S) or glutathione S-transferase to yield LTC_4. After carrier-mediated transport of LTB_4 and LTC_4 to the extracellular milieu, LTC_4 can be further metabolized to LTD_4 through the cleavage of glutamic acid from the glutathione moiety, and additional glycine cleavage yields LTE_4[28,33] (Figure 1).

5-LOX is expressed predominantly by neutrophils, eosinophils, monocytes, macrophages and mast cells. Although nonleukocytes express 5-LOX and FLAP to a lesser extent and are not believed to synthesize appreciable amounts of LTs, expression of LTA_4-H and/or LTC_4-S, uptake of exogenous LTA_4 and further metabolization is possible, *via* a process referred to as transcellular biosynthesis[34].

CysLT signaling is initiated upon binding of a li-

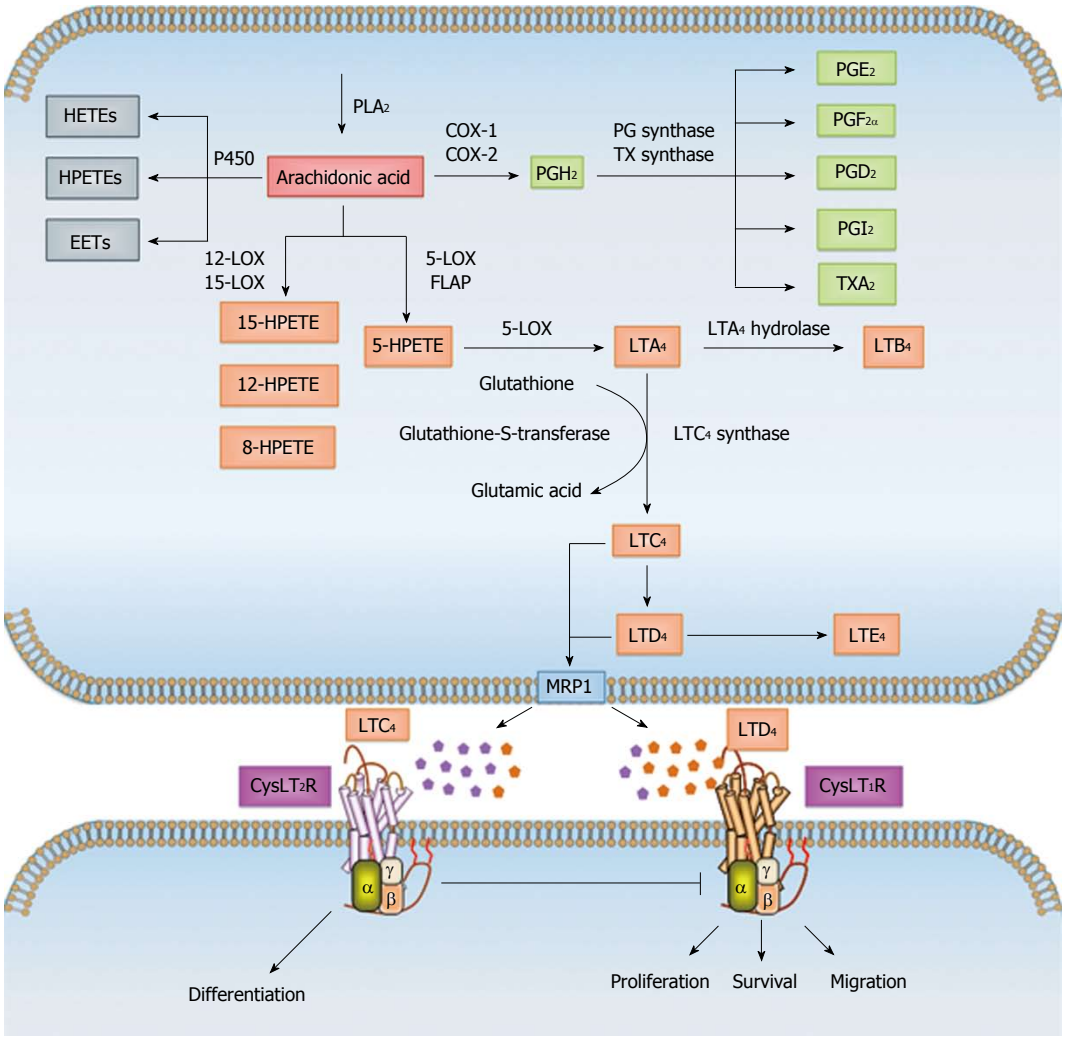


Figure 1 Overview of arachidonic acid metabolism. Arachidonic acid (AA) is a polyunsaturated fatty acid found in the phospholipids of cell membranes. AA is mobilized into the cytoplasm mostly by the activation of calcium-dependent cytosolic phospholipase A_2 (cPLA$_2$). Free AA in the cytosol can be enzymatically metabolized to eicosanoids through three major pathways: the cytochrome P450, cyclooxygenase (COX) and/or 5-lipoxygenase (5-LOX) pathways. In the P450 pathway, AA is metabolized to epoxyeicosatrienoic acids (EETs), hydroxyeicosatetraenoic acids (HETEs) and hydroperoxyeicosatetraenoic acids (HPETEs). In the COX pathway, AA is enzymatically converted to the intermediate prostaglandin H_2 (PGH$_2$), which is then sequentially metabolized to prostanoids, including prostaglandins (PGs), such as PGE$_2$, PGF$_2$, PGD$_2$ and PGI$_2$, and thromboxanes (TXs) such as TXA$_2$ by specific prostaglandin and thromboxane synthases. In the LOX pathway, AA is metabolized by 12- and 15-LOX to 8-, 12- and 15-HPETE or by 5-LOX and 5-lipoxygenase activating protein (FLAP) to intermediary 5-HPETE. 5-HPETE is further processed to form leukotrienes (LTs), the first of which is the unstable leukotriene A_4 (LTA$_4$). LTA$_4$ is subsequently converted to leukotriene B_4 (LTB$_4$) by LTA$_4$ hydrolase or together with glutathione to leukotriene C_4 (LTC$_4$) by LTC$_4$ synthase and glutathione-S-transferase. LTC$_4$ is converted by ubiquitous enzymes to form leukotriene D_4 (LTD$_4$) and leukotriene E_4 (LTE$_4$). The members of the multidrug resistance-associated protein (MRP) family are efflux transporters for both PGs and LTs. The cysteinyl leukotrienes (CysLTs) LTC$_4$, LTD$_4$ and LTE$_4$ act *via* G protein-coupled receptors CysLT$_1$R and CysLT$_2$R at the cell surface and induce different signaling mechanisms.

gand to one of the two G-protein-coupled receptors (GPCRs), CysLT$_1$R and CysLT$_2$R located at the plasma membrane[35,36], although the presence of other CysLT receptors such as GPR17, P2Y$_{12}$, and CysLT$_E$R have also been suggested[37-39]. Both CysLT$_1$R and CysLT$_2$R can also be localized to the nuclear membrane, since CysLT$_1$R has a bipartite nuclear localization sequence and CysLT$_2$R possesses an interferon regulatory 7 (IRF7) site, which in turn carries a nuclear localization sequence domain[40-42]. While the affinity of CysLT$_1$R for LTD$_4$ is high, the CysLT$_2$R has a low but an equal affinity for LTD$_4$ and LTC$_4$[35,36]. Functionally, CysLTs induce smooth muscle contraction, vascular leakage, eosinophil recruitment in inflammatory diseases, mucus production and chemotaxis[43-46].

LTB$_4$ also plays a pivotal role in inflammatory processes such as leukocyte chemoattraction, particularly of granulocytes and T cells, induction of rapid invasion and recruitment of these cells to the plasma membrane of endothelial cells, production of reactive oxygen species, and induction of gene expression[47,48]. LTB$_4$ mediates its signaling *via* two GPCRs: BLT$_1$ and BLT$_2$[49,50]. BLT$_1$ binds to LTB$_4$ with an affinity higher than that of the BLT$_2$ receptor. The tissue distribution of the two receptors is quite different. Whereas BLT$_1$ expression in both mice and humans has been reported to be predominantly restricted to peripheral leukocytes, BLT$_2$ expression in humans appears to be fairly ubiquitous, with the highest

level observed in the spleen, liver, and lymphocytes[51].

CYSTEINYL LEUKOTRIENES AND THEIR RECEPTORS IN COLORECTAL CANCER

IBD and colorectal cancer

Inflammation and CRC initiation and dissemination go hand in hand[10,52]. The most well-established connection exists between IBD-both UC and CD- and CRC[53-55]. "IBD" is a name given to a group of prolonged inflammatory disorders of the intestinal tract associated with debilitating symptoms and epithelial damage. The risk of developing CRC is 30%-50% higher in patients with IBD[56,57]. IBDs are characterized by increased leukocyte infiltration into the intestinal wall, where they can induce non-specific inflammation through activation and production of AA-derived pro-inflammatory metabolites such as LTs and PGs and subsequent tissue injury. Thus, the gastrointestinal tract is richly supplied with these eicosanoids that mediate several gastrointestinal diseases, including cancers. High levels of LTs such as LTE_4 have been detected in the urine of patients with UC and CD[58,59]. Among CysLTs, the presence of LTD_4 at an IBD site increases the risk of consequential cancer development, and specific LTD_4 antagonists have been shown to reduce colonic inflammation[60]. Although UC is fundamentally similar to CD, a few differences exist, primarily the presentation of a cytokine profile with a T helper 2 (Th2) antibody-mediated response[61]. CD is an autoimmune disease associated with T helper 1 (Th1)-mediated cytokines such as interleukin-12 (IL-12), IFN-γ and tumor necrosis factor-alpha (TNF-α)[61,62].

Colitis-associated cancer (CAC) is known to be highly infiltrated by several cells of the innate immune system, including neutrophils, mast cells, NKs, DCs and TAMs[63]. Moreover, recent evidence supports the concept that malignant tumors also recruit a specific subpopulation of myeloid cells called myeloid-derived suppressor cells[64]. These cells share some characteristics with monocytes, macrophages, neutrophils, and DCs and help suppress any potential anti-tumor immune response and tumor angiogenesis. As in several cancers, including CRC, in which the major inflammatory cellular components are macrophages, TAMs contribute immensely to cancer growth and expansion. TAMs are macrophages that display an M2 type (alternatively activated phenotype) and secrete high levels of Th2 cytokines, growth factors and inflammatory mediators that promote tumor growth, angiogenesis, and metastasis[65,66]. We have observed a high intra-tumoral density of TAMs in colon cancer tissue compared with the adjacent normal tissue, and M2 macrophages were required for effective colon cancer cell migration *via* factors derived from M2 macrophages and their association with signal regulatory protein alpha (SIRP-α) through CD47[67].

Eicosanoids and colorectal cancer

Apart from its role in inflammation-associated diseases such as asthma, psoriasis, rheumatoid arthritis and IBD[68], LTB_4 has pro-tumorigenic effects in breast cancer, melanoma, lymphoma, and head and neck carcinoma[69-72]. Increased expression of LTB_4 and its receptor BLT_1 have been demonstrated in human CRC tissue[73]. Ihara *et al*[73] demonstrated significant expression of BLT_1 in the colon cancer cell lines Caco-2 and HT-29. Using both the 5-LOX inhibitor AA-861 and selective BLT_1 antagonist U75302 in these cell lines, the authors showed induction of apoptosis and reduced proliferation[73]. LTB_4-stimulated extracellular signal-regulated kinase (Erk) activation in these cancer cells was also abrogated by U75302. A subsequent study investigated the effectiveness of another LTB_4 receptor antagonist (LY293111) in combination with gemcitabine, an anti-tumor adjuvant and radiosensitizer, on the proliferation rate of human colon cancer cell lines LoVo and HT-29 in an athymic heterotrophic xenograft mouse model and found a significant reduction in tumor growth due to apoptosis *via* the mitochondrial pathway[74]. The findings from these and several other studies emphasize the role of LTB_4 signaling in colon cancer cells and warrant the use of specific LTB_4 receptor antagonists to suppress CRC expansion.

Among the eicosanoids derived from the COX-pathway, PGE_2 is the most abundant and extensively studied in cancer, including CRC[29]. In both the spontaneous adenomatous polyposis coli $(Apc)^{Min/+}$ mouse model of intestinal cancer and the azoxymethane (AOM)-induced CRC mouse model, PGE_2 has been shown to increase the tumor burden[75,76]. Selective inhibition of PGE_2 synthesis, through genetic deletion of microsomal PGES (mPGES-1), significantly reduced tumor formation in an $Apc^{Min/+}$ and AOM-induced mouse model of intestinal and CRC, respectively, and further established the role of PGE_2 in tumorigenesis[77,78].

Increased expression of the enzymes responsible for production of PGs and LTs-COX-2 and 5-LOX, respectively has been documented in human colorectal adenocarcinomas compared with adjacent normal mucosa[79-81]. Various clinical trials over the past two decades have highlighted the use of eicosanoid-depressing and anti-inflammatory drugs in the prevention and treatment of CRC. Two groups of compounds have shown promising results: aspirin (NSAID) and celecoxib (COX-2 selective inhibitor)[82].

Cysteinyl leukotrienes, their receptors and colorectal cancer

Upregulated expression of $CysLT_1R$ has been observed in several human cancers, including transitional cell carcinoma (TCC) in the bladder, neuroblastomas, and brain, prostate, breast, and CRCs[6,80,83-86]. We have shown that high $CysLT_1R$ tumor expression is associated with a poor survival prognosis in breast and CRC patients[80,86], whereas concomitant low $CysLT_1R$ and high $CysLT_2R$ expression indicate a good prognosis in CRC patients[87]. We have also demonstrated high $CysLT_1R$ expression in established colon cancer cell lines[42,80]. The $CysLT_1R$ and

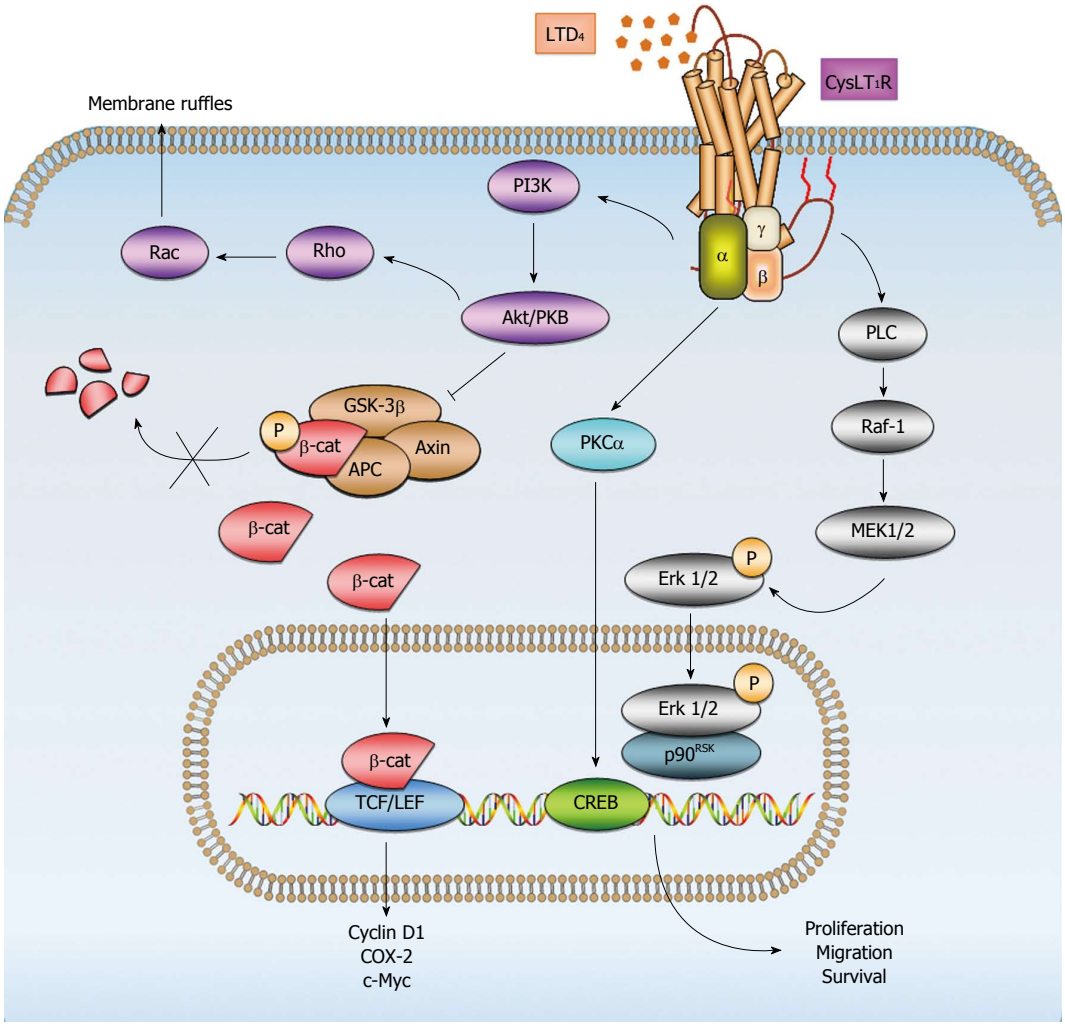


Figure 2 Signaling pathways of LTD_4 and $CysLT_1R$. Upon stimulation with LTD_4, various downstream signaling pathways become activated. LTD_4 induces cell membrane ruffles through phosphoinositide 3-kinase (PI3K) signaling, which in turn stimulates protein kinase Akt/PKB and the small GTPases Rho and Rac. Akt/PKB can inhibit glycogen synthase kinase-3 β (GSK-3β), which comprises the destruction complex for cytosolic β-catenin together with Axin and adenomatous polyposis coli (APC). Inhibition of the destruction complex leads to the accumulation of β-catenin in the cytosol and translocation to the nucleus. In the nucleus, β-catenin interacts primarily with members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors to activate target genes, leading to the expression of various proteins, such as cyclin D1, COX-2 and c-Myc, which contribute to diverse cellular processes, including proliferation and migration. LTD_4-$CysLT_1R$ signaling also regulates cell proliferation *via* mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (Erk) pathways. LTD_4-$CysLT_1R$ in turn activates phospholipase C (PLC), Raf-1 and mitogen-activated protein kinase kinase (MEK-1/2). This activation leads to the translocation of p-Erk from the cytosol to the nucleus and interaction with $p90^{RSK}$, which can activate various transcription factors. Conversely, LTD_4-$CysLT_1R$ mediates survival through protein kinase Cα (PKCα) and the transcription factor cAMP response element-binding protein (CREB). β-cat: β-catenin.

$CysLT_2R$ expression ratio seems to be important in the disease etiology of CRC. Accordingly, we have shown that these two receptors are co-localized and form hetero- and homodimers in a human intestinal epithelial cell line and that LTC_4 stimulation of $CysLT_2R$ negatively regulates the plasma membrane expression of $CysLT_1R$ by inducing internalization of the receptor heterodimer complex[88]. The expression of $CysLT_1R$ has also been positively correlated with the cell survival factors COX-2 and Bcl-x_L in tumor specimens from patients with CRC[80].

We have previously observed that LTD_4, *via* $CysLT_1R$, induces upregulation of proteins associated with CRC - such as COX-2, β-catenin, and Bcl-2 - in intestinal epithelial cells[89]. Upregulation of β-catenin expression was shown to be dependent on phosphoinositide 3 kinase (PI3K)-glycogen synthase kinase 3β (GSK-3β) signaling[30]. Moreover, our previous work has shown that LTD_4-induced $CysLT_1R$ signaling results in cell proliferation, survival, and migration through distinct signaling pathways. LTD_4-induced $CysLT_1R$-signaling through cAMP response element-binding protein (CREB) and p90 ribosomal s6 kinase ($p90^{RSK}$) was shown to induce survival and proliferation, respectively, while inducing migration *via* the PI3K-Rac signaling pathway[90,91] (Figure 2).

COX-2 expression has also been detected in various colon cancer cells[92], and we have shown that LTD_4 *via* $CysLT_1R$ enhances survival by activating the mitogen-activated protein kinase kinase (Mek)/Erk signaling pathway and increasing COX-2 and subsequent Bcl-2 expression in the colon cancer cell line Caco-2[93]. Furthermore, we have demonstrated that LTD_4 *via* $CysLT_1R$ induces

increased proliferation and migration in the colon cancer cell line HCT-116, probably *via* the GSK-3β/β-catenin pathway with subsequent increased transcription of the target genes *MYC* and *CCD1*[94]. By contrast, decreased expression of CysLT$_2$R has been observed in different colon cancer cell lines (Caco-2 and SW480) compared with an epithelial intestinal cell line, and LTC$_4$ stimulation of CysLT$_2$R has been shown to induce differentiation as demonstrated by increased intestinal alkaline phosphatase activity in Caco-2 cells[42]. In the same colon cancer cell line, anti-tumorigenic IFN-α was shown to induce CysLT$_2$R promoter activity and expression, whereas mitogenic epidermal growth factor (EGF) displayed the opposite effect, suppressing CysLT$_2$R promoter activity and expression. LTC$_4$-mediated CysLT$_2$R signaling suppressed EGF-induced cell migration, and IFN-α induced expression of the differentiation marker mucin-2 and alkaline phosphatase activity[95].

These results indicate potential pro- and anti-tumorigenic properties conveyed by CysLT$_1$R and CysLT$_2$R, respectively, in CRC.

LEUKOTRIENE RECEPTOR ANTAGONISTS AND LEUKOTRIENE SYNTHESIS INHIBITORS

CysLT$_1$R antagonists have been used in studies of inflammatory diseases such as rheumatoid arthritis and atherosclerosis[96,97]. The CysLT$_1$R antagonists pranlukast, zafirlukast and montelukast are commercially available and are currently in clinical use to treat asthmatic patients[98]. Emerging data suggest that the pro-inflammatory CysLTs might have an important role in solid tumors.

CysLT$_1$R antagonist treatment has been shown to inhibit tumor growth by inducing apoptosis in a variety of human urological cancer cell lines (*e.g.*, renal cell carcinoma, bladder cancer, prostate cancer, and testicular cancer)[99]. Montelukast has been shown to induce early apoptosis in a bladder transitional cell carcinoma (TCC) cell line, as well as in three different prostate cancer cell lines[6,83]. In addition, montelukast has been shown to induce the intrinsic apoptotic pathway, resulting in cleavage of caspases 3 and 9, and cell cycle arrest in neuroblastoma cell lines[84].

Studies in CysLT$_1$R-deficient mice have revealed its role in enhanced vascular permeability during an acute inflammatory response[100]. Pranlukast and montelukast have been shown to reduce vascular permeability by regulating vascular endothelial growth factor (VEGF) expression in allergen-induced asthmatic lungs of mice[101]. Furthermore, the two abovementioned CysLT$_1$R antagonists have been shown to inhibit the permeability of peripheral capillaries, thereby preventing tumor metastasis in a Lewis lung carcinoma metastasis model[102]. Proliferation and migration of endothelial cells are needed to form new vessels, a process required in cancer development. Montelukast has been shown to reduce LTD$_4$-CysLT$_1$R-mediated migration of the endothelial cell line EA.hy926 *via* the Erk1/2 pathway[103]. In line with aforementioned data, we have demonstrated in a nude mouse xenograft model of colon cancer that reduction of tumor growth can be accomplished with CysLT$_1$R antagonist treatment. The molecular mechanisms underlying the observed inhibition of tumor growth was attributed to the reduction in proliferation, induction of apoptosis and impairment of angiogenesis[104].

We have also shown that the CysLT$_1$R antagonist ZM198,615 reduces proliferation in the colon cancer cell lines Caco-2 and SW480[105]. Cianchi *et al*[106] reported the additive effects of the COX-2 selective inhibitor celecoxib, when combined with either the 5-LOX inhibitor MK886 or CysLT$_1$R antagonist LY171883, in reducing the proliferative ability of Caco-2 and HT29 cells. The combined treatment was also shown to induce apoptosis, whereas none of these compounds had any effect alone.

The COX pathway is the most extensively studied among eicosanoid pathways in CRC prevention and/or therapy. However, the cardiovascular side effects associated with long-term usage of NSAIDs and selective COX-2 inhibitors have raised some concerns. Other approaches are being explored, such as inhibition of 5-LOX activity. Simultaneous dual inhibition of COX-2 and 5-LOX activity could possibly provide a more effective and tolerable therapy than COX-inhibition alone. Accordingly, the anti-tumor effects of celecoxib in colon cancer cells were augmented when combined with inhibition of 5-LOX activity using the FLAP inhibitor MK886[106]. The combined inhibition of COX-2 and 5-LOX activity have also shown a more pronounced anti-tumor growth effect in a cigarette smoke-promoting mouse xenograft model of CRC[107]. However, targeting either the COX or LOX pathway alone resulted in a shunt toward the other pathway, except in the latter study, in which a shunt was observed when COX-2 activity was targeted with celecoxib. The abovementioned studies investigated the effects on CRC growth targeting the eicosanoid production in epithelial cells. However, the activity of 5-LOX of mast cells was also shown to be important in intestinal polyp formation in APCΔ^{468} mice. The mast cells were found to utilize 5-LOX to promote proliferation of intestinal epithelial cells and recruit myeloid-derived suppressor cells to the polyp site[108]. Another possible effective chemopreventive option against CRC could be the modification of AA metabolism. Apc$^{Min/+}$ mice with the fed diets containing highly purified ω-3 polyunsaturated fatty acids were shown to have their mucosal AA replaced, presumably with a reduction in the production of pro-inflammatory mediators. Reduced polyp formation could be observed in both the intestine and the colon of these mice. These effects were associated with significantly decreased proliferation, COX-2 expression, and nuclear β-catenin accumulation, as well as a concomitant increase in apoptosis in the intestinal epithelium[109]. A reduction in size and number of rectal polyps has also been observed in patients with hereditary CRC (familial adenomatous polyposis, FAP) who have undergone colectomy and received highly purified ω-3-polyunsaturated fatty acids[110].

CONCLUSION

Deregulated AA metabolism creates an imbalance in the tissue homeostatic events of proliferation, regeneration and repair, and host defense. Additionally, deregulated AA metabolism contributes to sustained inflammatory processes that could result in CRC development. Among the implicated inflammatory mediators are the eicosanoids, such as CysLTs. Thus, the modification of CysLT signaling could pave the way for the development of new personalized medicine for patients with CRC[104].

REFERENCES

1 **Tenesa A**, Dunlop MG. New insights into the aetiology of colorectal cancer from genome-wide association studies. *Nat Rev Genet* 2009; **10**: 353-358 [PMID: 19434079 DOI: 10.1038/nrg2574]

2 **Beller TC**, Friend DS, Maekawa A, Lam BK, Austen KF, Kanaoka Y. Cysteinyl leukotriene 1 receptor controls the severity of chronic pulmonary inflammation and fibrosis. *Proc Natl Acad Sci USA* 2004; **101**: 3047-3052 [PMID: 14970333 DOI: 10.1073/pnas.0400235101]

3 **Capra V**, Thompson MD, Sala A, Cole DE, Folco G, Rovati GE. Cysteinyl-leukotrienes and their receptors in asthma and other inflammatory diseases: critical update and emerging trends. *Med Res Rev* 2007; **27**: 469-527 [PMID: 16894531 DOI: 10.1002/med.20071]

4 **Riccioni G**, Bäck M, Capra V. Leukotrienes and atherosclerosis. *Curr Drug Targets* 2010; **11**: 882-887 [PMID: 20388065 DOI: 10.2174/138945010791320881]

5 **Schain F**, Schain D, Mahshid Y, Liu C, Porwit A, Xu D, Claesson HE, Sundström C, Björkholm M, Sjöberg J. Differential expression of cysteinyl leukotriene receptor 1 and 15-lipoxygenase-1 in non-Hodgkin lymphomas. *Clin Lymphoma Myeloma* 2008; **8**: 340-347 [PMID: 19064398 DOI: 10.3816/CLM.2008.n.049]

6 **Matsuyama M**, Funao K, Hayama T, Tanaka T, Kawahito Y, Sano H, Takemoto Y, Nakatani T, Yoshimura R. Relationship between cysteinyl-leukotriene-1 receptor and human transitional cell carcinoma in bladder. *Urology* 2009; **73**: 916-921 [PMID: 19167045 DOI: 10.1016/j.urology.2008.11.005]

7 **Sperling RI**. Eicosanoids in rheumatoid arthritis. *Rheum Dis Clin North Am* 1995; **21**: 741-758 [PMID: 8619097]

8 **Nicosia S**, Capra V, Rovati GE. Leukotrienes as mediators of asthma. *Pulm Pharmacol Ther* 2001; **14**: 3-19 [PMID: 11162414 DOI: 10.1006/pupt.2000.0262]

9 **Hendel J**, Nielsen OH. Expression of cyclooxygenase-2 mRNA in active inflammatory bowel disease. *Am J Gastroenterol* 1997; **92**: 1170-1173 [PMID: 9219792]

10 **Coussens LM**, Werb Z. Inflammation and cancer. *Nature* 2002; **420**: 860-867 [PMID: 12490959 DOI: 10.1038/nature0132]

11 **Rakoff-Nahoum S**. Why cancer and inflammation? *Yale J Biol Med* 2006; **79**: 123-130 [PMID: 17940622]

12 **Karin M**, Lawrence T, Nizet V. Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer. *Cell* 2006; **124**: 823-835 [PMID: 16497591 DOI: 10.1016/j.cell.2006.02.016]

13 **Balkwill F**, Mantovani A. Inflammation and cancer: back to Virchow? *Lancet* 2001; **357**: 539-545 [PMID: 11229684 DOI: 10.1016/S0140-6736(00)04046-0]

14 **Wang D**, Dubois RN. Eicosanoids and cancer. *Nat Rev Cancer* 2010; **10**: 181-193 [PMID: 20168319 DOI: 10.1038/nrc2809]

15 **Algra AM**, Rothwell PM. Effects of regular aspirin on long-term cancer incidence and metastasis: a systematic comparison of evidence from observational studies versus randomised trials. *Lancet Oncol* 2012; **13**: 518-527 [PMID: 22440112 DOI: 10.1016/S1470-2045(12)70112-2]

16 **Burn J**, Gerdes AM, Macrae F, Mecklin JP, Moeslein G, Olschwang S, Eccles D, Evans DG, Maher ER, Bertario L, Bisgaard ML, Dunlop MG, Ho JW, Hodgson SV, Lindblom A, Lubinski J, Morrison PJ, Murday V, Ramesar R, Side L, Scott RJ, Thomas HJ, Vasen HF, Barker G, Crawford G, Elliott F, Movahedi M, Pylvanainen K, Wijnen JT, Fodde R, Lynch HT, Mathers JC, Bishop DT. Long-term effect of aspirin on cancer risk in carriers of hereditary colorectal cancer: an analysis from the CAPP2 randomised controlled trial. *Lancet* 2011; **378**: 2081-2087 [PMID: 22036019 DOI: 10.1016/S0140-6736(11)61049-0]

17 **Moysich KB**, Menezes RJ, Ronsani A, Swede H, Reid ME, Cummings KM, Falkner KL, Loewen GM, Bepler G. Regular aspirin use and lung cancer risk. *BMC Cancer* 2002; **2**: 31 [PMID: 12453317 DOI: 10.1186/1471-2407-2-31]

18 **Cossack M**, Ghaffary C, Watson P, Snyder C, Lynch H. Aspirin Use is Associated with Lower Prostate Cancer Risk in Male Carriers of BRCA Mutations. *J Genet Couns* 2013; Epub ahead of print [PMID: 23881471 DOI: 10.1007/s10897-013-9629-8]

19 **Negus RP**, Stamp GW, Hadley J, Balkwill FR. Quantitative assessment of the leukocyte infiltrate in ovarian cancer and its relationship to the expression of C-C chemokines. *Am J Pathol* 1997; **150**: 1723-1734 [PMID: 9137096]

20 **Condeelis J**, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* 2006; **124**: 263-266 [PMID: 16439202 DOI: 10.1016/j.cell.2006.01.007]

21 **Egeblad M**, Littlepage LE, Werb Z. The fibroblastic coconspirator in cancer progression. *Cold Spring Harb Symp Quant Biol* 2005; **70**: 383-388 [PMID: 16869775 DOI: 10.1101/sqb.2005.70.007]

22 **Egeblad M**, Werb Z. New functions for the matrix metalloproteinases in cancer progression. *Nat Rev Cancer* 2002; **2**: 161-174 [PMID: 11990853 DOI: 10.1038/nrc745]

23 **Wang D**, Dubois RN. Prostaglandins and cancer. *Gut* 2006; **55**: 115-122 [PMID: 16118353 DOI: 10.1136/gut.2004.047100]

24 **Zlotnik A**. Chemokines and cancer. *Int J Cancer* 2006; **119**: 2026-2029 [PMID: 16671092 DOI: 10.1002/ijc.22024]

25 **Karin M**, Greten FR. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* 2005; **5**: 749-759 [PMID: 16175180 DOI: 10.1038/nri1703]

26 **Singh RK**, Gupta S, Dastidar S, Ray A. Cysteinyl leukotrienes and their receptors: molecular and functional characteristics. *Pharmacology* 2010; **85**: 336-349 [PMID: 20516735 DOI: 10.1159/000312669]

27 **Samuelsson B**. The discovery of the leukotrienes. *Am J Respir Crit Care Med* 2000; **161**: S2-S6 [PMID: 10673217 DOI: 10.1164/ajrccm.161.supplement_1.ltta-1]

28 **Peters-Golden M**, Henderson WR. Leukotrienes. *N Engl J Med* 2007; **357**: 1841-1854 [PMID: 17978293 DOI: 10.1056/NEJMra071371]

29 **Cathcart MC**, Lysaght J, Pidgeon GP. Eicosanoid signalling pathways in the development and progression of colorectal cancer: novel approaches for prevention/intervention. *Cancer Metastasis Rev* 2011; **30**: 363-385 [PMID: 22134655 DOI: 10.1007/s10555-011-9324-x]

30 **Mezhybovska M**, Wikström K, Ohd JF, Sjölander A. The inflammatory mediator leukotriene D4 induces beta-catenin signaling and its association with antiapoptotic Bcl-2 in intestinal epithelial cells. *J Biol Chem* 2006; **281**: 6776-6784 [PMID: 16407243 DOI: 10.1074/jbc.M509999200]

31 **Yudina Y**, Parhamifar L, Bengtsson AM, Juhas M, Sjölander A. Regulation of the eicosanoid pathway by tumour necrosis factor alpha and leukotriene D4 in intestinal epithelial cells. *Prostaglandins Leukot Essent Fatty Acids* 2008; **79**: 223-231 [PMID: 19042113 DOI: 10.1016/j.plefa.2008.09.024]

32 **Mezhybovska M**, Yudina Y, Abhyankar A, Sjölander A. Beta-catenin is involved in alterations in mitochondrial activ-

ity in non-transformed intestinal epithelial and colon cancer cells. *Br J Cancer* 2009; **101**: 1596-1605 [PMID: 19826421 DOI: 10.1038/sj.bjc.6605342]
33 **Lam BK**, Austen KF. Leukotriene C4 synthase: a pivotal enzyme in cellular biosynthesis of the cysteinyl leukotrienes. *Prostaglandins Other Lipid Mediat* 2002; **68-69**: 511-520 [PMID: 12432940]
34 **Fabre JE**, Goulet JL, Riche E, Nguyen M, Coggins K, Offenbacher S, Koller BH. Transcellular biosynthesis contributes to the production of leukotrienes during inflammatory responses in vivo. *J Clin Invest* 2002; **109**: 1373-1380 [PMID: 12021253 DOI: 10.1172/JCI14869]
35 **Lynch KR**, O'Neill GP, Liu Q, Im DS, Sawyer N, Metters KM, Coulombe N, Abramovitz M, Figueroa DJ, Zeng Z, Connolly BM, Bai C, Austin CP, Chateauneuf A, Stocco R, Greig GM, Kargman S, Hooks SB, Hosfield E, Williams DL, Ford-Hutchinson AW, Caskey CT, Evans JF. Characterization of the human cysteinyl leukotriene CysLT1 receptor. *Nature* 1999; **399**: 789-793 [PMID: 10391245 DOI: 10.1038/21658]
36 **Heise CE**, O'Dowd BF, Figueroa DJ, Sawyer N, Nguyen T, Im DS, Stocco R, Bellefeuille JN, Abramovitz M, Cheng R, Williams DL, Zeng Z, Liu Q, Ma L, Clements MK, Coulombe N, Liu Y, Austin CP, George SR, O'Neill GP, Metters KM, Lynch KR, Evans JF. Characterization of the human cysteinyl leukotriene 2 receptor. *J Biol Chem* 2000; **275**: 30531-30536 [PMID: 10851239 DOI: 10.1074/jbc.M003490200]
37 **Ciana P**, Fumagalli M, Trincavelli ML, Verderio C, Rosa P, Lecca D, Ferrario S, Parravicini C, Capra V, Gelosa P, Guerrini U, Belcredito S, Cimino M, Sironi L, Tremoli E, Rovati GE, Martini C, Abbracchio MP. The orphan receptor GPR17 identified as a new dual uracil nucleotides/cysteinyl-leukotrienes receptor. *EMBO J* 2006; **25**: 4615-4627 [PMID: 16990797 DOI: 10.1038/sj.emboj.7601341]
38 **Paruchuri S**, Tashimo H, Feng C, Maekawa A, Xing W, Jiang Y, Kanaoka Y, Conley P, Boyce JA. Leukotriene E4-induced pulmonary inflammation is mediated by the P2Y12 receptor. *J Exp Med* 2009; **206**: 2543-2555 [PMID: 19822647 DOI: 10.1084/jem.20091240]
39 **Maekawa A**, Kanaoka Y, Xing W, Austen KF. Functional recognition of a distinct receptor preferential for leukotriene E4 in mice lacking the cysteinyl leukotriene 1 and 2 receptors. *Proc Natl Acad Sci USA* 2008; **105**: 16695-16700 [PMID: 18931305 DOI: 10.1073/pnas.0808993105]
40 **Jans DA**, Xiao CY, Lam MH. Nuclear targeting signal recognition: a key control point in nuclear transport? *Bioessays* 2000; **22**: 532-544 [PMID: 10842307 DOI: 10.1002/(SICI)1521-1878(200006)]
41 **Servant MJ**, Tenoever B, Lin R. Overlapping and distinct mechanisms regulating IRF-3 and IRF-7 function. *J Interferon Cytokine Res* 2002; **22**: 49-58 [PMID: 11846975 DOI: 10.1089/107999002753452656]
42 **Magnusson C**, Ehrnström R, Olsen J, Sjölander A. An increased expression of cysteinyl leukotriene 2 receptor in colorectal adenocarcinomas correlates with high differentiation. *Cancer Res* 2007; **67**: 9190-9198 [PMID: 17909024 DOI: 10.1158/0008-5472.CAN-07-0771]
43 **Chan CC**, McKee K, Tagari P, Chee P, Ford-Hutchinson A. Eosinophil-eicosanoid interactions: inhibition of eosinophil chemotaxis in vivo by a LTD4-receptor antagonist. *Eur J Pharmacol* 1990; **191**: 273-280 [PMID: 1964905 DOI: 10.1016/0014-2999(90)94159-U]
44 **Barnes NC**, Piper PJ, Costello JF. Comparative effects of inhaled leukotriene C4, leukotriene D4, and histamine in normal human subjects. *Thorax* 1984; **39**: 500-504 [PMID: 6463929 DOI: 10.1136/thx.39.7.500]
45 **Drazen JM**, Austen KF, Lewis RA, Clark DA, Goto G, Marfat A, Corey EJ. Comparative airway and vascular activities of leukotrienes C-1 and D in vivo and in vitro. *Proc Natl Acad Sci USA* 1980; **77**: 4354-4358 [PMID: 6933488 DOI: 10.1073/pnas.77.7.4354]
46 **Marom Z**, Shelhamer JH, Bach MK, Morton DR, Kaliner M. Slow-reacting substances, leukotrienes C4 and D4, increase the release of mucus from human airways in vitro. *Am Rev Respir Dis* 1982; **126**: 449-451 [PMID: 7125334]
47 **Ford-Hutchinson AW**. Leukotriene B4 in inflammation. *Crit Rev Immunol* 1990; **10**: 1-12 [PMID: 2155000]
48 **Funk CD**. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 2001; **294**: 1871-1875 [PMID: 11729303 DOI: 10.1126/science.294.5548.1871]
49 **Yokomizo T**, Izumi T, Chang K, Takuwa Y, Shimizu T. A G-protein-coupled receptor for leukotriene B4 that mediates chemotaxis. *Nature* 1997; **387**: 620-624 [PMID: 9177352 DOI: 10.1038/42506]
50 **Yokomizo T**, Kato K, Terawaki K, Izumi T, Shimizu T. A second leukotriene B(4) receptor, BLT2. A new therapeutic target in inflammation and immunological disorders. *J Exp Med* 2000; **192**: 421-432 [PMID: 10934230 DOI: 10.1084/jem.192.3.421]
51 **Yokomizo T**, Izumi T, Shimizu T. Leukotriene B4: metabolism and signal transduction. *Arch Biochem Biophys* 2001; **385**: 231-241 [PMID: 11368003 DOI: 10.1006/abbi.2000.2168]
52 **Hussain SP**, Hofseth LJ, Harris CC. Radical causes of cancer. *Nat Rev Cancer* 2003; **3**: 276-285 [PMID: 12671666 DOI: 10.1038/nrc1046]
53 **Bernstein CN**, Blanchard JF, Kliewer E, Wajda A. Cancer risk in patients with inflammatory bowel disease: a population-based study. *Cancer* 2001; **91**: 854-862 [PMID: 11241255 DOI: 10.1002/1097-0142(20010215)]
54 **Hammerbeck DM**, Brown DR. Presence of immunocytes and sulfidopeptide leukotrienes in the inflamed guinea pig distal colon. *Inflammation* 1996; **20**: 413-425 [PMID: 8872504 DOI: 10.1007/BF01486743]
55 **Rubin DC**, Shaker A, Levin MS. Chronic intestinal inflammation: inflammatory bowel disease and colitis-associated colon cancer. *Front Immunol* 2012; **3**: 107 [PMID: 22586430 DOI: 10.3389/fimmu.2012.00107]
56 **Eaden J**. Review article: colorectal carcinoma and inflammatory bowel disease. *Aliment Pharmacol Ther* 2004; **20** Suppl 4: 24-30 [PMID: 15352890 DOI: 10.1111/j.1365-2036.2004.02046.x]
57 **Ekbom A**, Helmick C, Zack M, Adami HO. Ulcerative colitis and colorectal cancer. A population-based study. *N Engl J Med* 1990; **323**: 1228-1233 [PMID: 2215606 DOI: 10.1056/NEJM199011013231802]
58 **Sharon P**, Stenson WF. Enhanced synthesis of leukotriene B4 by colonic mucosa in inflammatory bowel disease. *Gastroenterology* 1984; **86**: 453-460 [PMID: 6319219]
59 **Kim JH**, Tagari P, Griffiths AM, Ford-Hutchinson A, Smith C, Sherman PM. Levels of peptidoleukotriene E4 are elevated in active Crohn's disease. *J Pediatr Gastroenterol Nutr* 1995; **20**: 403-407 [PMID: 7636682 DOI: 10.1097/00005176-199505000-00005]
60 **Nishikawa M**, Hikasa Y, Hori K, Tanida N, Shimoyama T. Effect of leukotriene C4D4 antagonist on colonic damage induced by intracolonic administration of trinitrobenzene sulfonic acid in rats. *J Gastroenterol* 1995; **30**: 34-40 [PMID: 7719412 DOI: 10.1007/BF01211372]
61 **Shanahan F**. Inflammatory bowel disease: immunodiagnostics, immunotherapeutics, and ecotherapeutics. *Gastroenterology* 2001; **120**: 622-635 [PMID: 11179240 DOI: 10.1053/gast.2001.22122]
62 **Fuss IJ**, Neurath M, Boirivant M, Klein JS, de la Motte C, Strong SA, Fiocchi C, Strober W. Disparate CD4+ lamina propria (LP) lymphokine secretion profiles in inflammatory bowel disease. Crohn's disease LP cells manifest increased secretion of IFN-gamma, whereas ulcerative colitis LP cells manifest increased secretion of IL-5. *J Immunol* 1996; **157**: 1261-1270 [PMID: 8757634]
63 **Atreya I**, Neurath MF. Immune cells in colorectal cancer: prognostic relevance and therapeutic strategies. *Expert Rev*

Anticancer Ther 2008; **8**: 561-572 [PMID: 18402523 DOI: 10.1586/14737140.8.4.561]
64 **Gabrilovich DI**, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol* 2009; **9**: 162-174 [PMID: 19197294 DOI: 10.1038/nri2506]
65 **Solinas G**, Germano G, Mantovani A, Allavena P. Tumor-associated macrophages (TAM) as major players of the cancer-related inflammation. *J Leukoc Biol* 2009; **86**: 1065-1073 [PMID: 19741157 DOI: 10.1189/jlb.0609385]
66 **Sica A**, Schioppa T, Mantovani A, Allavena P. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. *Eur J Cancer* 2006; **42**: 717-727 [PMID: 16520032 DOI: 10.1016/j.ejca.2006.01.003]
67 **Zhang Y**, Sime W, Juhas M, Sjölander A. Crosstalk between colon cancer cells and macrophages via inflammatory mediators and CD47 promotes tumour cell migration. *Eur J Cancer* 2013; **49**: 3320-3334 [PMID: 23810249 DOI: 10.1016/j.ejca.2013.06.005]
68 **Sampson AP**. The role of eosinophils and neutrophils in inflammation. *Clin Exp Allergy* 2000; **30** Suppl 1: 22-27 [PMID: 10849470 DOI: 10.1046/j.1365-2222.2000.00092]
69 **Earashi M**, Noguchi M, Tanaka M. In vitro effects of eicosanoid synthesis inhibitors in the presence of linoleic acid on MDA-MB-231 human breast cancer cells. *Breast Cancer Res Treat* 1996; **37**: 29-37 [PMID: 8750525 DOI: 10.1007/BF01806629]
70 **Okano-Mitani H**, Ikai K, Imamura S. Human melanoma cells generate leukotrienes B4 and C4 from leukotriene A4. *Arch Dermatol Res* 1997; **289**: 347-351 [PMID: 9209681 DOI: 10.1007/s004030050203]
71 **Bittner S**, Wielckens K. Glucocorticoid-induced lymphoma cell growth inhibition: the role of leukotriene B4. *Endocrinology* 1988; **123**: 991-1000 [PMID: 2840273 DOI: 10.1210/endo-123-2-991]
72 **el-Hakim IE**, Langdon JD, Zakrzewski JT, Costello JF. Leukotriene B4 and oral cancer. *Br J Oral Maxillofac Surg* 1990; **28**: 155-159 [PMID: 1966928 DOI: 10.1016/0266-4356(90)90078-Y]
73 **Ihara A**, Wada K, Yoneda M, Fujisawa N, Takahashi H, Nakajima A. Blockade of leukotriene B4 signaling pathway induces apoptosis and suppresses cell proliferation in colon cancer. *J Pharmacol Sci* 2007; **103**: 24-32 [PMID: 17220595 DOI: 10.1254/jphs.FP0060651]
74 **Hennig R**, Ding XZ, Tong WG, Witt RC, Jovanovic BD, Adrian TE. Effect of LY293111 in combination with gemcitabine in colonic cancer. *Cancer Lett* 2004; **210**: 41-46 [PMID: 15172119 DOI: 10.1016/j.canlet.2004.02.023]
75 **Wang D**, Wang H, Shi Q, Katkuri S, Walhi W, Desvergne B, Das SK, Dey SK, DuBois RN. Prostaglandin E(2) promotes colorectal adenoma growth via transactivation of the nuclear peroxisome proliferator-activated receptor delta. *Cancer Cell* 2004; **6**: 285-295 [PMID: 15380519 DOI: 10.1016/j.ccr.2004.08.011]
76 **Kawamori T**, Uchiya N, Sugimura T, Wakabayashi K. Enhancement of colon carcinogenesis by prostaglandin E2 administration. *Carcinogenesis* 2003; **24**: 985-990 [PMID: 12771044 DOI: 10.1093/carcin/bgg033]
77 **Nakanishi M**, Montrose DC, Clark P, Nambiar PR, Belinsky GS, Claffey KP, Xu D, Rosenberg DW. Genetic deletion of mPGES-1 suppresses intestinal tumorigenesis. *Cancer Res* 2008; **68**: 3251-3259 [PMID: 18451151 DOI: 10.1158/0008-5472.CAN-07-6100]
78 **Nakanishi M**, Menoret A, Tanaka T, Miyamoto S, Montrose DC, Vella AT, Rosenberg DW. Selective PGE(2) suppression inhibits colon carcinogenesis and modifies local mucosal immunity. *Cancer Prev Res (Phila)* 2011; **4**: 1198-1208 [PMID: 21576350 DOI: 10.1158/1940-6207]
79 **Dreyling KW**, Hoppe U, Peskar BA, Morgenroth K, Kozuschek W, Peskar BM. Leukotriene synthesis by human gastrointestinal tissues. *Biochim Biophys Acta* 1986; **878**: 184-193 [PMID: 3019409 DOI: 10.1016/0005-2760(86)90145-1]
80 **Ohd JF**, Nielsen CK, Campbell J, Landberg G, Löfberg H, Sjölander A. Expression of the leukotriene D4 receptor CysLT1, COX-2, and other cell survival factors in colorectal adenocarcinomas. *Gastroenterology* 2003; **124**: 57-70 [PMID: 12512030 DOI: 10.1053/gast.2003.50011]
81 **Soumaoro LT**, Iida S, Uetake H, Ishiguro M, Takagi Y, Higuchi T, Yasuno M, Enomoto M, Sugihara K. Expression of 5-lipoxygenase in human colorectal cancer. *World J Gastroenterol* 2006; **12**: 6355-6360 [PMID: 17072961]
82 **Wang D**, DuBois RN. The role of anti-inflammatory drugs in colorectal cancer. *Annu Rev Med* 2013; **64**: 131-144 [PMID: 23020877 DOI: 10.1146/annurev-med-112211-154330]
83 **Matsuyama M**, Hayama T, Funao K, Kawahito Y, Sano H, Takemoto Y, Nakatani T, Yoshimura R. Overexpression of cysteinyl LT1 receptor in prostate cancer and CysLT1R antagonist inhibits prostate cancer cell growth through apoptosis. *Oncol Rep* 2007; **18**: 99-104 [PMID: 17549353]
84 **Sveinbjörnsson B**, Rasmuson A, Baryawno N, Wan M, Pettersen I, Ponthan F, Orrego A, Haeggström JZ, Johnsen JI, Kogner P. Expression of enzymes and receptors of the leukotriene pathway in human neuroblastoma promotes tumor survival and provides a target for therapy. *FASEB J* 2008; **22**: 3525-3536 [PMID: 18591367 DOI: 10.1096/fj.07-103457]
85 **Zhang WP**, Hu H, Zhang L, Ding W, Yao HT, Chen KD, Sheng WW, Chen Z, Wei EQ. Expression of cysteinyl leukotriene receptor 1 in human traumatic brain injury and brain tumors. *Neurosci Lett* 2004; **363**: 247-251 [PMID: 15182953 DOI: 10.1016/j.neulet.2004.03.088]
86 **Magnusson C**, Liu J, Ehrnström R, Manjer J, Jirström K, Andersson T, Sjölander A. Cysteinyl leukotriene receptor expression pattern affects migration of breast cancer cells and survival of breast cancer patients. *Int J Cancer* 2011; **129**: 9-22 [PMID: 20824707 DOI: 10.1002/ijc.25648]
87 **Magnusson C**, Mezhybovska M, Lörinc E, Fernebro E, Nilbert M, Sjölander A. Low expression of CysLT1R and high expression of CysLT2R mediate good prognosis in colorectal cancer. *Eur J Cancer* 2010; **46**: 826-835 [PMID: 20064706 DOI: 10.1016/j.ejca.2009]
88 **Parhamifar L**, Sime W, Yudina Y, Vilhardt F, Mörgelin M, Sjölander A. Ligand-induced tyrosine phosphorylation of cysteinyl leukotriene receptor 1 triggers internalization and signaling in intestinal epithelial cells. *PLoS One* 2010; **5**: e14439 [PMID: 21203429 DOI: 10.1371/journal.pone.0014439]
89 **Ohd JF**, Wikström K, Sjölander A. Leukotrienes induce cell-survival signaling in intestinal epithelial cells. *Gastroenterology* 2000; **119**: 1007-1018 [PMID: 11040187 DOI: 10.1053/gast.2000.18141]
90 **Paruchuri S**, Sjölander A. Leukotriene D4 mediates survival and proliferation via separate but parallel pathways in the human intestinal epithelial cell line Int 407. *J Biol Chem* 2003; **278**: 45577-45585 [PMID: 12912998 DOI: 10.1074/jbc.M302881200]
91 **Paruchuri S**, Broom O, Dib K, Sjölander A. The pro-inflammatory mediator leukotriene D4 induces phosphatidylinositol 3-kinase and Rac-dependent migration of intestinal epithelial cells. *J Biol Chem* 2005; **280**: 13538-13544 [PMID: 15657050 DOI: 10.1074/jbc.M409811200]
92 **Parker J**, Kaplon MK, Alvarez CJ, Krishnaswamy G. Prostaglandin H synthase expression is variable in human colorectal adenocarcinoma cell lines. *Exp Cell Res* 1997; **236**: 321-329 [PMID: 9344613 DOI: 10.1006/excr.1997.3741]
93 **Wikström K**, Ohd JF, Sjölander A. Regulation of leukotriene-dependent induction of cyclooxygenase-2 and Bcl-2. *Biochem Biophys Res Commun* 2003; **302**: 330-335 [PMID: 12604350 DOI: 10.1016/S0006-]
94 **Salim T**, Sand-Dejmek J, Sjölander A. The inflammatory mediator leukotriene D4 induces subcellular β-catenin translocation and migration of colon cancer cells. *Exp Cell Res* 2013; Epub ahead of print [PMID: 24211746 DOI: 10.1016/

j.yexcr.2013.10.021]
95 **Magnusson C**, Bengtsson AM, Liu M, Liu J, Ceder Y, Ehrnström R, Sjölander A. Regulation of cysteinyl leukotriene receptor 2 expression--a potential anti-tumor mechanism. *PLoS One* 2011; **6**: e29060 [PMID: 22194989 DOI: 10.1371/journal.pone.0029060]
96 **Shiota N**, Shimoura K, Okunishi H. Pathophysiological role of mast cells in collagen-induced arthritis: study with a cysteinyl leukotriene receptor antagonist, montelukast. *Eur J Pharmacol* 2006; **548**: 158-166 [PMID: 16949072 DOI: 10.1016/j.ejphar.2006.07.046]
97 **Mueller CF**, Wassmann K, Widder JD, Wassmann S, Chen CH, Keuler B, Kudin A, Kunz WS, Nickenig G. Multidrug resistance protein-1 affects oxidative stress, endothelial dysfunction, and atherogenesis via leukotriene C4 export. *Circulation* 2008; **117**: 2912-2918 [PMID: 18506003 DOI: 10.1161/CIRCULATIONAHA.107.747667]
98 **Bateman ED**, Hurd SS, Barnes PJ, Bousquet J, Drazen JM, FitzGerald M, Gibson P, Ohta K, O'Byrne P, Pedersen SE, Pizzichini E, Sullivan SD, Wenzel SE, Zar HJ. Global strategy for asthma management and prevention: GINA executive summary. *Eur Respir J* 2008; **31**: 143-178 [PMID: 18166595 DOI: 10.1183/09031936.00138707]
99 **Matsuyama M**, Yoshimura R. Cysteinyl-leukotriene1 receptor is a potent target for the prevention and treatment of human urological cancer. *Mol Med Rep* 2010; **3**: 245-251 [PMID: 21472229 DOI: 10.3892/mmr_00000247]
100 **Maekawa A**, Austen KF, Kanaoka Y. Targeted gene disruption reveals the role of cysteinyl leukotriene 1 receptor in the enhanced vascular permeability of mice undergoing acute inflammatory responses. *J Biol Chem* 2002; **277**: 20820-20824 [PMID: 11932261 DOI: 10.1074/jbc.M203163200]
101 **Lee KS**, Kim SR, Park HS, Jin GY, Lee YC. Cysteinyl leukotriene receptor antagonist regulates vascular permeability by reducing vascular endothelial growth factor expression. *J Allergy Clin Immunol* 2004; **114**: 1093-1099 [PMID: 15536415 DOI: 10.1016/j.jaci.2004.07.039]
102 **Nozaki M**, Yoshikawa M, Ishitani K, Kobayashi H, Houkin K, Imai K, Ito Y, Muraki T. Cysteinyl leukotriene receptor antagonists inhibit tumor metastasis by inhibiting capillary permeability. *Keio J Med* 2010; **59**: 10-18 [PMID: 20375653 DOI: 10.2302/kjm.59.10]
103 **Yuan YM**, Fang SH, Qian XD, Liu LY, Xu LH, Shi WZ, Zhang LH, Lu YB, Zhang WP, Wei EQ. Leukotriene D4 stimulates the migration but not proliferation of endothelial cells mediated by the cysteinyl leukotriene cyslt(1) receptor via the extracellular signal-regulated kinase pathway. *J Pharmacol Sci* 2009; **109**: 285-292 [PMID: 19234368 DOI: 10.1254/jphs.08321FP]
104 **Savari S**, Liu M, Zhang Y, Sime W, Sjölander A. CysLT(1)R antagonists inhibit tumor growth in a xenograft model of colon cancer. *PLoS One* 2013; **8**: e73466 [PMID: 24039952 DOI: 10.1371/journal.pone.0073466]
105 **Paruchuri S**, Mezhybovska M, Juhas M, Sjölander A. Endogenous production of leukotriene D4 mediates autocrine survival and proliferation via CysLT1 receptor signalling in intestinal epithelial cells. *Oncogene* 2006; **25**: 6660-6665 [PMID: 16715140 DOI: 10.1038/sj.onc.1209666]
106 **Cianchi F**, Cortesini C, Magnelli L, Fanti E, Papucci L, Schiavone N, Messerini L, Vannacci A, Capaccioli S, Perna F, Lulli M, Fabbroni V, Perigli G, Bechi P, Masini E. Inhibition of 5-lipoxygenase by MK886 augments the antitumor activity of celecoxib in human colon cancer cells. *Mol Cancer Ther* 2006; **5**: 2716-2726 [PMID: 17121918 DOI: 10.1158/1535-7163.MCT-06-0318]
107 **Ye YN**, Wu WK, Shin VY, Bruce IC, Wong BC, Cho CH. Dual inhibition of 5-LOX and COX-2 suppresses colon cancer formation promoted by cigarette smoke. *Carcinogenesis* 2005; **26**: 827-834 [PMID: 15637091 DOI: 10.1093/carcin/bgi012]
108 **Cheon EC**, Khazaie K, Khan MW, Strouch MJ, Krantz SB, Phillips J, Blatner NR, Hix LM, Zhang M, Dennis KL, Salabat MR, Heiferman M, Grippo PJ, Munshi HG, Gounaris E, Bentrem DJ. Mast cell 5-lipoxygenase activity promotes intestinal polyposis in APCDelta468 mice. *Cancer Res* 2011; **71**: 1627-1636 [PMID: 21216893 DOI: 10.1158/0008-5472.CAN-10-1923]
109 **Fini L**, Piazzi G, Ceccarelli C, Daoud Y, Belluzzi A, Munarini A, Graziani G, Fogliano V, Selgrad M, Garcia M, Gasbarrini A, Genta RM, Boland CR, Ricciardiello L. Highly purified eicosapentaenoic acid as free fatty acids strongly suppresses polyps in Apc(Min/+) mice. *Clin Cancer Res* 2010; **16**: 5703-5711 [PMID: 21030497 DOI: 10.1158/1078-0432.CCR-10-1990]
110 **West NJ**, Clark SK, Phillips RK, Hutchinson JM, Leicester RJ, Belluzzi A, Hull MA. Eicosapentaenoic acid reduces rectal polyp number and size in familial adenomatous polyposis. *Gut* 2010; **59**: 918-925 [PMID: 20348368 DOI: 10.1136/gut.2009.200642]

P- Reviewers: Depoortere I, He C **S- Editor**: Qi Y
L- Editor: A **E- Editor**: Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.978

World J Gastroenterol 2014 January 28; 20(4): 978-987
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Accumulation of aberrant DNA methylation during colorectal cancer development

Eiji Sakai, Atsushi Nakajima, Atsushi Kaneda

Eiji Sakai, Atsushi Nakajima, Department of Gastroenterology, Yokohama City University School of Medicine, Yokohama 236-0027, Japan
Eiji Sakai, Atsushi Kaneda, Genome Science Division, Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo 113-8654, Japan
Atsushi Kaneda, Department of Molecular Oncology, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan
Atsushi Kaneda, CREST, Japan Science and Technology Agency, Saitama 332-0012, Japan
Author contributions: Sakai E and Kaneda A wrote the manuscript; Nakajima A and Kaneda A supervised the study.
Correspondence to: Atsushi Kaneda, MD, PhD, Department of Molecular Oncology, Graduate School of Medicine, Chiba University, Inohana 1-8-1, Chuo-ku, Chiba-City 260-8670, Japan. kaneda@chiba-u.jp
Telephone: +81-43-2262039 Fax: +81-43-2262039
Received: September 28, 2013 Revised: November 12, 2013
Accepted: December 12, 2013
Published online: January 28, 2014

Abstract

Despite the recent advances in the therapeutic modalities, colorectal cancer (CRC) remains to be one of the most common causes of cancer-related death. CRC arises through accumulation of multiple genetic and epigenetic alterations that transform normal colonic epithelium into adenocarcinomas. Among crucial roles of epigenetic alterations, gene silencing by aberrant DNA methylation of promoter regions is one of the most important epigenetic mechanisms. Recent comprehensive methylation analyses on genome-wide scale revealed that sporadic CRC can be classified into distinct epigenotypes. Each epigenotype cooperates with specific genetic alterations, suggesting that they represent different molecular carcinogenic pathways. Precursor lesions of CRC, such as conventional and serrated adenomas, already show similar methylation accumulation to CRC, and can therefore be classified into those epigenotypes of CRC. In addition, specific DNA methylation already occurs in the normal colonic mucosa, which might be utilized for prediction of the personal CRC risk. DNA methylation is suggested to occur at an earlier stage than carcinoma formation, and may predict the molecular basis for future development of CRC. Here, we review DNA methylation and CRC classification, and discuss the possible clinical usefulness of DNA methylation as biomarkers for the diagnosis, prediction of the prognosis and the response to therapy of CRC.

Key words: Colorectal cancer; Colorectal adenoma; Aberrant crypt foci; Genetic mutation; Epigenotype; DNA methylation; Colorectal carcinogenesis

Core tip: Colorectal cancer (CRC) is a heterogeneous disease which involves several distinct molecular carcinogenetic pathways. Recent comprehensive genome-wide analyses clarify detailed DNA methylation statuses of cancer-related genes in CRC. We and others have investigated the association between DNA methylation and genetic alterations, and performed classification of CRC/their precursors, including conventional adenomas, serrated adenomas, non-polypoid colorectal neoplasms and aberrant crypt foci. In addition, we also evaluated the usefulness of DNA methylation markers as surrogate biomarkers for diagnosis, prognosis and therapeutic application of CRC. Here, we review the DNA methylation status and classification of CRC to understand the roles of DNA methylation in colorectal carcinogenesis.

Sakai E, Nakajima A, Kaneda A. Accumulation of aberrant DNA methylation during colorectal cancer development. *World J Gastroenterol* 2014; 20(4): 978-987 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/978.htm DOI: http://

dx.doi.org/10.3748/wjg.v20.i4.978

INTRODUCTION

Colorectal cancer (CRC) arises through accumulation of multiple acquired genetic and epigenetic alterations that cause malignant transformation of normal colonic epithelium to adenocarcinoma[1,2]. These carcinogenetic processes were first described in the model of the adenoma-carcinoma sequence[3], and somatic mutations of tumor-suppressor genes (*e.g.*, *APC*, *p53* and *DCC*) and activating mutations in the *KRAS* oncogene are well-known genetic alterations involved in this model[3-5] (Table 1).

CRC can be biologically divided into those with microsatellite instability (MSI), characterized by DNA replication and repair defects, and those with chromosomal instability (CIN), characterized by aneuploidy, multiple chromosomal rearrangements and accumulation of somatic mutations in oncogenes[6]. These genomic instabilities have been reported to be closely associated with the molecular heterogeneity of CRC[7], which is a factor responsible for the significant variability in the prognosis and treatment response among patients with the same stage of CRC[8]. Since the distinct molecular subtypes of CRC are difficult to be accurately distinguished histologically or clinically, technologies that can detect significant molecular alterations in CRC on genome-wide scale had been expected to be developed. Recently, exome sequencing analyses revealed the involvement of many somatic mutations of genes, including *SMAD4*, *FBXW7*, *TCF7L2*, and *FAM123B*[9-11]. Although hundreds of mutations, on average, are found in genomes of CRC, only a small set of functionally important genes are proposed to be involved with cancer formation as driver genes in individual cancer[11]. Whereas key mutational changes are necessary for the initiation and progression of CRC, the number of genes silenced by epigenetic mechanisms is greater than the number of genetic mutations in CRC[11], suggesting a crucial role of epigenetic alterations.

Recently, we and other groups performed epigenotyping of CRC, by unsupervised hierarchical clustering method using comprehensive and quantitative methylation data (Table 2). These results demonstrated that CRC can be clearly clustered into three DNA methylation epigenotypes[12-14]. Interestingly, each of the epigenotypes showed a unique association with a variety of genetic mutations (*i.e.*, *BRAF*, *KRAS* and *TP53*) and the genomic instability status of sporadic CRC, indicating that they develop through distinct carcinogenetic pathways. Moreover, the intermediate-methylation subtype with *KRAS* mutation showed a poorer prognosis than other subtypes, suggesting that the DNA methylation status could be used as prognostic markers. Subsequently, we conducted epigenotyping of conventional adenomas and serrated adenomas, and showed that these precursor lesions of CRC can also be classified into the three epigenotypes[15]. These findings suggested that epigenotype development occur at an earlier stage than carcinoma formation, and is already completed at the adenoma stage. In this review, we focus on the importance of DNA methylation and provide an overview of the classification of CRC and their precursors, and discuss the clinical applications of aberrant DNA methylation as biomarkers for the diagnosis, prediction of the prognosis and the response to therapy of CRC.

ABERRANT DNA METHYLATION ON GENE PROMOTERS

Two major types of epigenetic alterations closely linked to CRC are aberrant DNA methylation and covalent histone modifications[16]. Gene silencing by aberrant DNA methylation of its promoter region is one of the most important epigenetic mechanisms to inactivate the expression of tumor-suppressor genes. While the majority of CpG sites in the genome are known to be methylated in normal mammalian cells, unmethylated CpG sites are typically present in the genomic regions known as CpG islands. CpG islands are reported to overlap the promoter regions in 60%-70% of genes and tend to be protected from methylation; however they can be aberrantly methylated during the carcinogenetic process[17]. Investigation of genes using aberrant methylation as markers is useful to identify novel tumor-suppressor genes and methylation markers for cancer classification[18-23]. Therefore, several methods for genome-wide analysis have been developed since the 1990's[24-28]. These epigenetic alterations have been noted to play crucial roles not only in cancer progression, but also in cancer initiation, since the alterations have been identified in the pre-cancerous "normal" tissues that could modify cancer risk[2,29-31]. Recently, genome-wide DNA methylation analysis tools have been developed to reveal the detailed epigenetic backgrounds of CRC[12-14]. Importantly, gene silencing resulting from aberrant DNA methylation cooperates with other genetic mechanisms to alter the key molecular pathways critical in colorectal carcinogenesis[29] (Figure 1).

CLASSIFICATION OF CRC USING DNA METHYLATION INFORMATION

CpG island methylator phenotype

In 1999, Toyota *et al*[21] reported that some CRCs show a significantly high frequency of aberrant DNA methylation in specific CpG islands, named CpG island methylator phenotype (CIMP). CIMP-positive CRC shows DNA hypermethylation at a specific subset of genomic loci[32,33] and is highly enriched for an activating mutation of *BRAF*[34,35]. Hypermethylation of CpG islands in gene promoter regions results in transcriptional repression. For example, CIMP-mediated gene silencing of the mismatch repair gene *MLH1* by promoter hypermethylation is the molecular basis for MSI in sporadic microsatellite-unstable CRC, and most sporadic microsatellite-unstable

Table 1 Colorectal carcinogenic pathways and genetic alterations

	MSI	Methylation	KRAS	BRAF	TP53	Reports
Adenoma-carcinoma sequence	-	+/-	++	-	+	Grady *et al*[1] Vogelstein *et al*[3]
Serrated pathway	+	++	+	++	+/-	Howkins *et al*[32] Weisenberger *et al*[35]
De novo pathway	-	-	+	-	-	Yashiro *et al*[58] Kinney *et al*[59]

MSI: Microsatellite instability.

Table 2 Reports on colorectal cancer classification by methylation information

Ref.	Marker selection	Methylation analysis methods	Classification method	Methylation phenotypes
Toyota *et al*[20]	Genome-wide (MCA-RDA)	COBRA	Methlation frequency	CIMP+ CIMP-
Weisenberger *et al*[35]	MethyLight markers	MethyLight	Hierarchical clustering	CIMP+ CIMP-
Ogino *et al*[39]	Reported markers	MethyLight	Methylation frequency	CIMP-high CIMP-low CIMP-0
Shen *et al*[13]	Reported markers	Pyrosequence COBRA MCA MSP	Hierarchical clustering	CIMP1 CIMP2 CIMP-negative
Yagi *et al*[14]	Genome-wide (MeDIP-chip)	MassARRAY	Hierarchical clustering	HME IME LME
Hinoue *et al*[12]	Genome-wide (Infinium 27k)	MethyLight	Hierarchical clustering	CIMP-H CIMP-L Non-CIMP

CIMP: CpG island methylator phenotype; MCA: Methylated CpG island amplication; RDA: Representation difference analysis; COBRA: Combined bisulfite restriction analysis; MSP: Methylation-specific PCR; MeDIP: Methylated DNA immunoprecipitation; LME: Low-methylation epigenotype; IME: Intermediate-epigenotype; HME: High-methylation epigenotype.

CRC are therefore CIMP-positive[36]. CIMP-positive CRC inversely correlates with CRC with CIN[37,38], tends to occur in the proximal colon, and is commonly observed in women[32], suggesting that they appear to develop distinct carcinogenetic pathway from CIMP-negative CRC.

DNA methylation markers and CRC epigenotypes

Since the first CIMP markers were identified by Toyota *et al*[20,21], many other CIMP markers have been described, *e.g.*, *MLH1*, *NEUROG1*, *SOCS1*, *RUNX3*, *IGF2* and *CACNA1G*[18-22,35]. Using quantitative real-time PCR, Ogino *et al*[39] selected five CIMP markers to distinguish high from low levels of CIMP-mediated gene promoter methylation, and found that CIMP-low CRC tends to be associated with male sex and *KRAS* mutations. CIMP-low appears to be independent of the MSI status, suggesting that CIMP-low might be a different subtype of CRC from CIMP-high and CIMP-0. However, no clear difference was observed between CIMP-low and CIMP-0, because these methylation markers were specific for CIMP-high and not ideal for identification of the CIMP-low subtype. Sensitive and specific markers for CIMP-low were needed to be determined.

According to the results of unsupervised two-way hierarchical clustering based on the quantitative DNA methylation data of 27 previously reported gene promoter and genetic alterations, including mutations of *BRAF*, *KRAS*, and *p53*, Shen *et al*[13] proposed that CRC can be classified into three subsets, CIMP1, CIMP2, and CIMP-negative. This report successfully showed the existence of three clusters of CRC with different molecular characteristics: (1) CIMP1 with MSI-high (80%), *BRAF* mutation (53%) and high-methylation; (2) CIMP2 with *KRAS* mutation (92%) and different methylation; and (3) CIMP-negative with *p53* mutation (71%) and absence of these methylations. Integrated genetic and epigenetic analysis was found to be important, and genetic markers performed better than epigenetic markers in their classification of CRC[13].

To clarify whether CRC can be classified into more than two subsets using information on methylation accumulation alone, we performed comprehensive two-way unsupervised hierarchical clustering, using quantitative methylation data of genome-widely selected novel markers that were established through MeDIP-chip analysis. We demonstrated that CRC can be clearly classified into three distinct epigenotypes: high-, intermediate-, and low-methylation epigenotypes (HME, IME, and LME). HME was strongly correlated to the presence of the *BRAF* mutation (71%) and MSI-high (76%), and IME was

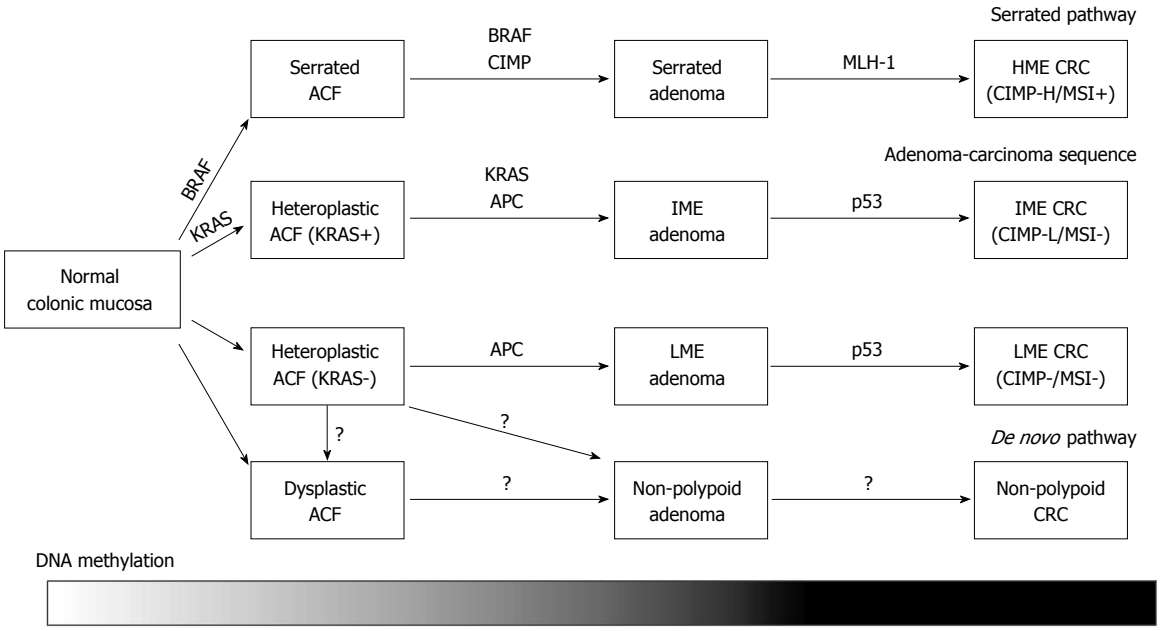


Figure 1 Carcinogenetic pathway and classification of colorectal cancer. Colorectal cancer (CRC) arises through accumulation of multiple acquired genetic and epigenetic alterations. Adenomas and CRCs can be classified into several sub-groups based on the status of DNA methylation and associated genetic mutations, suggesting that the different types of CRC developed through different molecular carcinogenetic pathways. DNA methylation accumulation occurs during aberrant cell expansion and is usually completed at adenoma stage. Non-polypoid colorectal neoplasms are hypothesized to develop through de novo pathway, whereas the epigenetic features of laterally spreading tumors have not yet been fully investigated.

strongly correlated to the presence of the *KRAS* mutation (63%)[14]. In our analysis, *p53* mutation was absent in HME, but was detected in both IME and LME. It was noteworthy that the methylation markers were clustered into two groups: (1) Group-1 markers included most of the known CIMP markers and showed methylation specifically in HME CRC; and (2) Group-2 markers including novel methylation markers which showed methylation in both HME and IME. It was also noteworthy that patients with IME *KRAS*-mutation(+) CRC showed a significantly worse prognosis.

Subsequent to our report, Hinoue *et al*[12] performed DNA methylation profiling of CRC using Illumina Infinium DNA methylation beadarray, and reported that CRC can be classified into three distinct epigenotypes (CIMP-H, CIMP-L and Non-CIMP), consistent with previous reports[13,14]. Genetic and epigenetic features of CIMP-H/CIMP-L CRC are also in agreement with those observed in the CIMP1/CIMP2 CRC[13] and the HME/IME CRC[14]. According to the frequency of *p53* mutation, they proposed that non-CIMP CRC could be classified into two distinct sub-groups; one with a significantly higher frequency of *p53* mutations (65%) and frequent occurrence in the distal colon, and the other with absence of both cancer-specific DNA methylation and gene mutations, and more frequent occurrence in the rectum.

CLASSIFICATION OF PRECURSOR LESIONS OF CRC

Genetic and epigenetic alterations of serrated adenomas

The majority of sporadic CRC is thought to develop from conventional adenomas through the adenoma-carcinoma sequence[3], whereas the serrated pathway has been considered as an alternative pathway distinct from the adenoma-carcinoma sequence. The serrated pathway is known to involve mutation of *BRAF*, *MLH1* methylation and CIMP[34,35]. While serrated adenomas are commonly CIMP-high and carry the *BRAF* mutation[34,40-45], conventional adenomas rarely exhibit these genetic and epigenetic alterations[40]. In addition, the risk factors for CIMP-high serrated adenomas are reported to be similar to those of CRC with CIMP[40]. Serrated adenoma is therefore considered to be a precursor of CIMP-positive CRC. Although CIMP-high and *BRAF* mutation were frequently observed at the adenoma stage, the prevalence of *MLH1* methylation was lower than that of CRC with CIMP[40-42,45]. Interestingly, *MLH1* methylation was more frequently observed in proximal, large serrated adenomas[40], suggesting that *MLH1* methylation is a late event in the serrated pathway, and heralds the transition from serrated adenoma to CRC, involving the mutator phenotype.

Epigenotype of conventional adenomas

While the genetic and epigenetic features among conventional and serrated adenomas have been demonstrated to be widely different, existence of DNA methylation phenotypes within conventional adenomas and their correlation to genetic mutations were not fully investigated. We investigated whether conventional adenomas could be classified into epigenotypes, and our CRC classification markers successfully classified conventional adenomas into two distinct epigenotypes, IME and LME[15]. There were no remarkable differences in the morphological and

pathological features among the two epigenotypes. While IME adenomas showed a significantly high frequency of the *KRAS* mutation (62%), LME adenomas did not show any genetic alterations, similar to the case of LME CRC. Interestingly, there was no difference in the methylation level between IME adenoma and IME cancer, suggesting that accumulation of aberrant DNA methylation is mostly completed at the adenoma stage. This indicated that additional aberration(s) other than DNA methylation are needed for adenomas to transform into CRC. The progression of adenomas to CRC is postulated to be associated with *p53* abnormalities[3], and DNA methylation of some genes, *e.g.*, *MGMT*, *CXLC12*, *TIMP3*, *ID4* and *IRF8*, might also be involved in the development to CRC[46,47].

Genetic and epigenetic alterations of non-polypoid colorectal neoplasms

Non-polypoid colorectal neoplasms that do not exhibit a macroscopic protruding appearance have been documented not only in Japan[48-53], but also in western countries[54,55]. They are characterized by lateral extensions along the luminal wall with a low vertical axis, and such tumors with a diameter of > 10 mm are called laterally spreading tumors (LSTs)[48]. The incidence of genetic alterations such as *KRAS*, *BRAF* and *p53*[49,56-58] and MSI[59] were less common in these non-polypoid colorectal neoplasms than those in conventional adenomas. In addition, a high percentage of these lesions are reported to exhibit high-grade dysplasia and rapidly invade the submucosal layer despite their small sizes[50-52,60]. Therefore, non-polypoid colorectal neoplasms are hypothesized to develop through an alternative carcinogenetic pathway (*i.e.*, *de novo* pathway) different from the adenoma-carcinoma sequence and the serrated pathway. LSTs are usually categorized into two subtypes based on their macroscopic morphology: the granular type and non-granular type[48]. Whereas the epigenetic features of LST have not yet been fully investigated, Hiraoka *et al*[61] reported frequent methylation of CIMP-markers and frequent *KRAS* mutation in the granular type, but not in the non-granular type. LST might be composed of several subtypes which exhibit distinct molecular pathway, and further investigations are needed to reveal the genetic and epigenetic features of nonpolypoid colorectal neoplasms and their association with colorectal carcinogenesis.

CLASSIFICATION OF ABERRANT CRYPT FOCI

Aberrant crypt foci and colorectal carcinogenesis

Aberrant crypt foci (ACF) are microscopic mucosal abnormalities, a subset of which postulated to be the earliest precursors of CRC[62]. ACF show increased expression of proliferative markers[63], and a significant correlation has been reported to exist between the presence of ACF and synchronous advanced neoplasia[62,64-71], suggesting a positive role of these lesions in colorectal carcinogenesis. Therefore, ACF have been recognized as a useful surrogate biomarker for CRC surveillance[72] and been used in recent chemoprevention trials[73-77]. Histopathologically, human ACF can be sub-classified into two categories: dysplastic and heteroplastic[62]. Dysplastic ACF resemble adenomas and sometimes lack mucin production[78], and are more common in familial adenomatous polyposis (FAP) patients than in sporadic CRC patients[79]. In contrast, heteroplastic ACF resemble hyperplastic polyps and lack dysplasia, and are highly identified in sporadic CRC patients.

Genetic and epigenetic alterations in aberrant crypt foci

Although all ACF from FAP patients carry the *APC* mutation[79], both the dysplastic and heteroplastic ACF from sporadic CRC patients frequently carry *KRAS* mutation, but not the *APC* mutation[79-81]. While *BRAF* mutation has rarely been identified in ACF[79,82], Rosenberg *et al*[83] reported that heteroplastic ACF with serrated pathology exclusively exhibit *BRAF* mutation. Although there was a report that CIMP-high was less frequently observed in ACF in sporadic CRC patients[80], the methylation status of ACF has not been well investigated. In our DNA methylation analysis in heteroplastic ACF, ACF showed frequent *KRAS* mutation, consist with previous reports. The levels of aberrant DNA methylation were significantly lower compared to adenomas[82], suggesting that DNA methylation accumulation might be requested during aberrant cell expansion in adenoma formation, but not in ACF formation.

DNA METHYLATION IN APPARENTLY NORMAL MUCOSA

Some of genes showing aberrant methylation in CRC, such as *ESR1*, *IGF2* and *TUSC3* are also methylated in histologically normal colonic epithelium. Aberrant DNA methylation of these genes is considered to increase in an age-dependent manner, and approximately half of them have also been shown to be involved in the pathogenesis of CRC[84-86].

The concept of "field cancerization" was proposed to explain the multiple primary lesions, local recurrence and increased susceptibility of normal tissue to malignant transformation[87]. The field changes occur at the molecular level, and these abnormalities of the normal colonic epithelium could be potential biomarkers for assessing the personal risk for future CRC development. Suzuki *et al*[88] reported that a higher incidence of hypermethylation and down-regulation of the *SFRP* genes, negative regulators of the WNT signaling pathway, were observed in the normal colonic mucosa from patients with CRC, than in that from patients without CRC. In addition, Kawakami *et al*[89] reported that higher methylation levels of age-related markers, such as *ESR1* and *MYOD*, were observed in the normal colonic mucosa from patients with CIMP-positive CRC than in that from patients without CRC. It was reported, in contrast, that lower methylation levels

of these markers were observed in the normal colonic mucosa from patients with CRC than from patients without CRC[90,91]. Recently, genome-wide DNA methylation analysis revealed that the gene methylation levels involved in the metabolic pathways of carbohydtates, lipids and amino acids were significantly different among normal colonic mucosa specimens obtained from patients with and without CRC[92]. While DNA methylation accumulation is expected to contribute to field cancerization in the colon, further studies are necessary to establish useful surrogate biomarkers for CRC surveillance.

CLINICAL APPLICATION OF DNA METHYLATION MARKERS

Early CRC detection could contribute to a reduction of CRC-related mortality. However, strategies such as colonoscopy are invasive, whereas the less-invasive fecal blood test shows low sensitivity and specificity[93]. Identification of noninvasively testable, high-quality biomarkers for CRC is therefore necessary. Recent genome-wide analyses were conducted to identify candidate DNA methylation markers for early CRC detection, by comparing the DNA methylation levels between CRC and/or adenomas, and matched normal colonic mucosa[93-96]. For example, Mori *et al*[94] reported that the methylation status of *VSX2* showed a high discriminative accuracy (83% sensitivity and 92% specificity). These potential biomarkers may allow reliable discrimination of CRC patients from tumor-free patients. Several clinical studies have been carried out to confirm the usefulness of stool and blood DNA-based methylation markers for early CRC detection[97,98]. In any application, classification marker genes are specifically methylated in some epigenotypes, therefore, genes that are commonly methylated in all CRCs, regardless of the epigenotype, would be useful markers for early CRC detection.

The MSI status has been proposed as a biomarker for determination of the prognosis and/or the effectiveness of FU chemotherapy in advanced CRC patients[99]. *KRAS* mutation in advanced CRC has been reported to be associated with a poor prognosis[100], and the usefulness of determining its presence for predicting a lack of response to EGFR-targeted therapy is well proven[101]. Our previous study revealed that IME CRC with *KRAS* mutation is associated with a poor prognosis. DNA methylation biomarkers for prediction of the therapeutic responses of CRC, however, have not been identified yet. Additional studies are needed to establish methylation biomarkers for application in clinical practice, *e.g.*, for prediction of the prognosis and of the responses to therapy of CRC.

CONCLUSION

Recent comprehensive genome-wide methylation analyses revealed that sporadic CRC can be classified into three distinct epigenotypes. Each of these CRC epigenotypes cooperates with specific genetic alterations, suggesting that they develop through different molecular carcinogenetic pathways. Serrated adenomas are commonly CIMP-high and carry *BRAF* mutation, thus postulated to be precursor lesions of CIMP-positive, MSI-high proximal CRCs. *MLH1* methylation has been suggested to be a late event in the serrated pathway, and heralds the transition from serrated adenoma to CIMP-positive CRC. Conventional adenomas can also be classified into two distinct epigenotypes. DNA methylation accumulation is mostly completed by the adenoma stage, and conventional adenomas are hypothesized to be precursors of CIMP1/IME/CIMP-low and CIMP0/LME/Non-CIMP CRCs. ACF showed significantly lower methylation levels than adenomas, suggesting that DNA methylation accumulation is a prerequisite for aberrant cell expansion in adenoma formation, but not in the formation of ACF.

DNA methylation may predict the molecular basis of CRC, and these markers might be present as useful surrogate markers for the diagnosis, prediction of the prognosis and the response to therapy of CRC. Some genes already showed aberrant methylation in apparently normal colonic mucosa, and their methylation may be related to field cancerization of CRC and predict cancer risk. Continued efforts to investigate the associations between molecular mechanisms of CRC and genetic/epigenetic alterations may allow us to understand colorectal carcinogenesis, and lead to the translation of these insights into clinical practice.

REFERENCES

1 **Grady WM**, Carethers JM. Genomic and epigenetic instability in colorectal cancer pathogenesis. *Gastroenterology* 2008; **135**: 1079-1099 [PMID: 18773902 DOI: 10.1053/j.gastro.2008.07.076]

2 **Kaneda A**, Feinberg AP. Loss of imprinting of IGF2: a common epigenetic modifier of intestinal tumor risk. *Cancer Res* 2005; **65**: 11236-11240 [PMID: 16357124 DOI: 10.1158/0008-5472.can-05-2959]

3 **Vogelstein B**, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL. Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988; **319**: 525-532 [PMID: 2841597 DOI: 10.1056/nejm198809013190901]

4 **Jen J**, Kim H, Piantadosi S, Liu ZF, Levitt RC, Sistonen P, Kinzler KW, Vogelstein B, Hamilton SR. Allelic loss of chromosome 18q and prognosis in colorectal cancer. *N Engl J Med* 1994; **331**: 213-221 [PMID: 8015568 DOI: 10.1056/nejm199407283310401]

5 **White RL**. Tumor suppressing pathways. *Cell* 1998; **92**: 591-592 [PMID: 9506511]

6 **Kinzler KW**, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996; **87**: 159-170 [PMID: 8861899]

7 **Dunican DS**, McWilliam P, Tighe O, Parle-McDermott A, Croke DT. Gene expression differences between the microsatellite instability (MIN) and chromosomal instability (CIN) phenotypes in colorectal cancer revealed by high-density cDNA array hybridization. *Oncogene* 2002; **21**: 3253-3257 [PMID: 12082642 DOI: 10.1038/sj.onc.1205431]

8 **Siegel R**, DeSantis C, Virgo K, Stein K, Mariotto A, Smith T, Cooper D, Gansler T, Lerro C, Fedewa S, Lin C, Leach C, Cannady RS, Cho H, Scoppa S, Hachey M, Kirch R, Jemal A, Ward E. Cancer treatment and survivorship statistics, 2012. *CA Cancer J Clin* 2012; **62**: 220-241 [PMID: 22700443 DOI:

10.3322/caac.21149]
9 **Cancer Genome Atlas Network**. Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 2012; **487**: 330-337 [PMID: 22810696 DOI: 10.1038/nature11252]
10 **Greenman C**, Stephens P, Smith R, Dalgliesh GL, Hunter C, Bignell G, Davies H, Teague J, Butler A, Stevens C, Edkins S, O'Meara S, Vastrik I, Schmidt EE, Avis T, Barthorpe S, Bhamra G, Buck G, Choudhury B, Clements J, Cole J, Dicks E, Forbes S, Gray K, Halliday K, Harrison R, Hills K, Hinton J, Jenkinson A, Jones D, Menzies A, Mironenko T, Perry J, Raine K, Richardson D, Shepherd R, Small A, Tofts C, Varian J, Webb T, West S, Widaa S, Yates A, Cahill DP, Louis DN, Goldstraw P, Nicholson AG, Brasseur F, Looijenga L, Weber BL, Chiew YE, DeFazio A, Greaves MF, Green AR, Campbell P, Birney E, Easton DF, Chenevix-Trench G, Tan MH, Khoo SK, Teh BT, Yuen ST, Leung SY, Wooster R, Futreal PA, Stratton MR. Patterns of somatic mutation in human cancer genomes. *Nature* 2007; **446**: 153-158 [PMID: 17344846 DOI: 10.1038/nature05610]
11 **Sjöblom T**, Jones S, Wood LD, Parsons DW, Lin J, Barber TD, Mandelker D, Leary RJ, Ptak J, Silliman N, Szabo S, Buckhaults P, Farrell C, Meeh P, Markowitz SD, Willis J, Dawson D, Willson JK, Gazdar AF, Hartigan J, Wu L, Liu C, Parmigiani G, Park BH, Bachman KE, Papadopoulos N, Vogelstein B, Kinzler KW, Velculescu VE. The consensus coding sequences of human breast and colorectal cancers. *Science* 2006; **314**: 268-274 [PMID: 16959974 DOI: 10.1126/science.1133427]
12 **Hinoue T**, Weisenberger DJ, Lange CP, Shen H, Byun HM, Van Den Berg D, Malik S, Pan F, Noushmehr H, van Dijk CM, Tollenaar RA, Laird PW. Genome-scale analysis of aberrant DNA methylation in colorectal cancer. *Genome Res* 2012; **22**: 271-282 [PMID: 21659424 DOI: 10.1101/gr.117523.110]
13 **Shen L**, Toyota M, Kondo Y, Lin E, Zhang L, Guo Y, Hernandez NS, Chen X, Ahmed S, Konishi K, Hamilton SR, Issa JP. Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. *Proc Natl Acad Sci USA* 2007; **104**: 18654-18659 [PMID: 18003927 DOI: 10.1073/pnas.0704652104]
14 **Yagi K**, Akagi K, Hayashi H, Nagae G, Tsuji S, Isagawa T, Midorikawa Y, Nishimura Y, Sakamoto H, Seto Y, Aburatani H, Kaneda A. Three DNA methylation epigenotypes in human colorectal cancer. *Clin Cancer Res* 2010; **16**: 21-33 [PMID: 20028768]
15 **Yagi K**, Takahashi H, Akagi K, Matsusaka K, Seto Y, Aburatani H, Nakajima A, Kaneda A. Intermediate methylation epigenotype and its correlation to KRAS mutation in conventional colorectal adenoma. *Am J Pathol* 2012; **180**: 616-625 [PMID: 22115708 DOI: 10.1016/j.ajpath.2011.10.010]
16 **Jones PA**, Baylin SB. The epigenomics of cancer. *Cell* 2007; **128**: 683-692 [PMID: 17320506 DOI: 10.1016/j.cell.2007.01.029]
17 **Bird A**. DNA methylation patterns and epigenetic memory. *Genes Dev* 2002; **16**: 6-21 [PMID: 11782440 DOI: 10.1101/gad.947102]
18 **Kaneda A**, Kaminishi M, Yanagihara K, Sugimura T, Ushijima T. Identification of silencing of nine genes in human gastric cancers. *Cancer Res* 2002; **62**: 6645-6650 [PMID: 12438262]
19 **Kaneda A**, Wakazono K, Tsukamoto T, Watanabe N, Yagi Y, Tatematsu M, Kaminishi M, Sugimura T, Ushijima T. Lysyl oxidase is a tumor suppressor gene inactivated by methylation and loss of heterozygosity in human gastric cancers. *Cancer Res* 2004; **64**: 6410-6415 [PMID: 15374948 DOI: 10.1158/0008-5472.can-04-1543]
20 **Toyota M**, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci USA* 1999; **96**: 8681-8686 [PMID: 10411935]
21 **Toyota M**, Ho C, Ahuja N, Jair KW, Li Q, Ohe-Toyota M, Baylin SB, Issa JP. Identification of differentially methylated sequences in colorectal cancer by methylated CpG island amplification. *Cancer Res* 1999; **59**: 2307-2312 [PMID: 10344734]
22 **Ushijima T**. Detection and interpretation of altered methylation patterns in cancer cells. *Nat Rev Cancer* 2005; **5**: 223-231 [PMID: 15719030 DOI: 10.1038/nrc1571]
23 **Ushijima T**, Morimura K, Hosoya Y, Okonogi H, Tatematsu M, Sugimura T, Nagao M. Establishment of methylation-sensitive-representational difference analysis and isolation of hypo- and hypermethylated genomic fragments in mouse liver tumors. *Proc Natl Acad Sci USA* 1997; **94**: 2284-2289 [PMID: 9122186]
24 **Gu H**, Bock C, Mikkelsen TS, Jäger N, Smith ZD, Tomazou E, Gnirke A, Lander ES, Meissner A. Genome-scale DNA methylation mapping of clinical samples at single-nucleotide resolution. *Nat Methods* 2010; **7**: 133-136 [PMID: 20062050 DOI: 10.1038/nmeth.1414]
25 **Hayashi H**, Nagae G, Tsutsumi S, Kaneshiro K, Kozaki T, Kaneda A, Sugisaki H, Aburatani H. High-resolution mapping of DNA methylation in human genome using oligonucleotide tiling array. *Hum Genet* 2007; **120**: 701-711 [PMID: 17024368 DOI: 10.1007/s00439-006-0254-6]
26 **Laird PW**. Principles and challenges of genomewide DNA methylation analysis. *Nat Rev Genet* 2010; **11**: 191-203 [PMID: 20125086 DOI: 10.1038/nrg2732]
27 **Lister R**, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM, Edsall L, Antosiewicz-Bourget J, Stewart R, Ruotti V, Millar AH, Thomson JA, Ren B, Ecker JR. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature* 2009; **462**: 315-322 [PMID: 19829295 DOI: 10.1038/nature08514]
28 **Weber M**, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, Schübeler D. Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. *Nat Genet* 2005; **37**: 853-862 [PMID: 16007088 DOI: 10.1038/ng1598]
29 **Baylin SB**, Ohm JE. Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? *Nat Rev Cancer* 2006; **6**: 107-116 [PMID: 16491070 DOI: 10.1038/nrc1799]
30 **Feinberg AP**, Ohlsson R, Henikoff S. The epigenetic progenitor origin of human cancer. *Nat Rev Genet* 2006; **7**: 21-33 [PMID: 16369569 DOI: 10.1038/nrg1748]
31 **Sakatani T**, Kaneda A, Iacobuzio-Donahue CA, Carter MG, de Boom Witzel S, Okano H, Ko MS, Ohlsson R, Longo DL, Feinberg AP. Loss of imprinting of Igf2 alters intestinal maturation and tumorigenesis in mice. *Science* 2005; **307**: 1976-1978 [PMID: 15731405 DOI: 10.1126/science.1108080]
32 **Hawkins N**, Norrie M, Cheong K, Mokany E, Ku SL, Meagher A, O'Connor T, Ward R. CpG island methylation in sporadic colorectal cancers and its relationship to microsatellite instability. *Gastroenterology* 2002; **122**: 1376-1387 [PMID: 11984524]
33 **Whitehall VL**, Wynter CV, Walsh MD, Simms LA, Purdie D, Pandeya N, Young J, Meltzer SJ, Leggett BA, Jass JR. Morphological and molecular heterogeneity within nonmicrosatellite instability-high colorectal cancer. *Cancer Res* 2002; **62**: 6011-6014 [PMID: 12414620]
34 **Kambara T**, Simms LA, Whitehall VL, Spring KJ, Wynter CV, Walsh MD, Barker MA, Arnold S, McGivern A, Matsubara N, Tanaka N, Higuchi T, Young J, Jass JR, Leggett BA. BRAF mutation is associated with DNA methylation in serrated polyps and cancers of the colorectum. *Gut* 2004; **53**: 1137-1144 [PMID: 15247181 DOI: 10.1136/gut.2003.037671]
35 **Weisenberger DJ**, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D, Koh H, Simms L, Barker M, Leggett B, Levine J, Kim M, French AJ, Thibodeau SN, Jass J, Haile R, Laird PW. CpG island methylator phenotype underlies

sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. *Nat Genet* 2006; **38**: 787-793 [PMID: 16804544 DOI: 10.1038/ng1834]
36 **Samowitz WS**. The CpG island methylator phenotype in colorectal cancer. *J Mol Diagn* 2007; **9**: 281-283 [PMID: 17591925]
37 **Cheng YW**, Pincas H, Bacolod MD, Schemmann G, Giardina SF, Huang J, Barral S, Idrees K, Khan SA, Zeng Z, Rosenberg S, Notterman DA, Ott J, Paty P, Barany F. CpG island methylator phenotype associates with low-degree chromosomal abnormalities in colorectal cancer. *Clin Cancer Res* 2008; **14**: 6005-6013 [PMID: 18829479 DOI: 10.1158/1078-0432.ccr-08-0216]
38 **Goel A**, Nagasaka T, Arnold CN, Inoue T, Hamilton C, Niedzwiecki D, Compton C, Mayer RJ, Goldberg R, Bertagnolli MM, Boland CR. The CpG island methylator phenotype and chromosomal instability are inversely correlated in sporadic colorectal cancer. *Gastroenterology* 2007; **132**: 127-138 [PMID: 17087942 DOI: 10.1053/j.gastro.2006.09.018]
39 **Ogino S**, Kawasaki T, Kirkner GJ, Loda M, Fuchs CS. CpG island methylator phenotype-low (CIMP-low) in colorectal cancer: possible associations with male sex and KRAS mutations. *J Mol Diagn* 2006; **8**: 582-588 [PMID: 17065427 DOI: 10.2353/jmoldx.2006.060082]
40 **Burnett-Hartman AN**, Newcomb PA, Potter JD, Passarelli MN, Phipps AI, Wurscher MA, Grady WM, Zhu LC, Upton MP, Makar KW. Genomic aberrations occurring in subsets of serrated colorectal lesions but not conventional adenomas. *Cancer Res* 2013; **73**: 2863-2872 [PMID: 23539450 DOI: 10.1158/0008-5472.can-12-3462]
41 **Kim YH**, Kakar S, Cun L, Deng G, Kim YS. Distinct CpG island methylation profiles and BRAF mutation status in serrated and adenomatous colorectal polyps. *Int J Cancer* 2008; **123**: 2587-2593 [PMID: 18798261 DOI: 10.1002/ijc.23840]
42 **Maeda T**, Suzuki K, Togashi K, Nokubi M, Saito M, Tsujinaka S, Kamiyama H, Konishi F. Sessile serrated adenoma shares similar genetic and epigenetic features with microsatellite unstable colon cancer in a location-dependent manner. *Exp Ther Med* 2011; **2**: 695-700 [PMID: 22977561 DOI: 10.3892/etm.2011.249]
43 **O'Brien MJ**, Yang S, Mack C, Xu H, Huang CS, Mulcahy E, Amorosino M, Farraye FA. Comparison of microsatellite instability, CpG island methylation phenotype, BRAF and KRAS status in serrated polyps and traditional adenomas indicates separate pathways to distinct colorectal carcinoma end points. *Am J Surg Pathol* 2006; **30**: 1491-1501 [PMID: 17122504 DOI: 10.1097/01.pas.0000213313.36306.85]
44 **Rex DK**, Ahnen DJ, Baron JA, Batts KP, Burke CA, Burt RW, Goldblum JR, Guillem JG, Kahi CJ, Kalady MF, O'Brien MJ, Odze RD, Ogino S, Parry S, Snover DC, Torlakovic EE, Wise PE, Young J, Church J. Serrated lesions of the colorectum: review and recommendations from an expert panel. *Am J Gastroenterol* 2012; **107**: 1315-129; quiz 1314, 1330 [PMID: 22710576 DOI: 10.1038/ajg.2012.161]
45 **Vaughn CP**, Wilson AR, Samowitz WS. Quantitative evaluation of CpG island methylation in hyperplastic polyps. *Mod Pathol* 2010; **23**: 151-156 [PMID: 19855373 DOI: 10.1038/modpathol.2009.150]
46 **Kim MS**, Lee J, Sidransky D. DNA methylation markers in colorectal cancer. *Cancer Metastasis Rev* 2010; **29**: 181-206 [PMID: 20135198 DOI: 10.1007/s10555-010-9207-6]
47 **Qi J**, Zhu YQ, Luo J, Tao WH. Hypermethylation and expression regulation of secreted frizzled-related protein genes in colorectal tumor. *World J Gastroenterol* 2006; **12**: 7113-7117 [PMID: 17131472]
48 **Kudo S**, Kashida H, Nakajima T, Tamura S, Nakajo K. Endoscopic diagnosis and treatment of early colorectal cancer. *World J Surg* 1997; **21**: 694-701 [PMID: 9276699]
49 **Minamoto T**, Sawaguchi K, Mai M, Yamashita N, Sugimura T, Esumi H. Infrequent K-ras activation in superficial-type (flat) colorectal adenomas and adenocarcinomas. *Cancer Res* 1994; **54**: 2841-2844 [PMID: 8187064]
50 **Minamoto T**, Sawaguchi K, Ohta T, Itoh T, Mai M. Superficial-type adenomas and adenocarcinomas of the colon and rectum: a comparative morphological study. *Gastroenterology* 1994; **106**: 1436-1443 [PMID: 8194688]
51 **Tada S**, Iida M, Matsumoto T, Yao T, Aoyagi K, Koga H, Tanoue Y, Fujishima M. Small flat cancer of the rectum: clinicopathologic and endoscopic features. *Gastrointest Endosc* 1995; **42**: 109-113 [PMID: 7590044]
52 **Watari J**, Saitoh Y, Obara T, Fujiya M, Maemoto A, Ayabe T, Ashida T, Yokota K, Orii Y, Kohgo Y. Natural history of colorectal nonpolypoid adenomas: a prospective colonoscopic study and relation with cell kinetics and K-ras mutations. *Am J Gastroenterol* 2002; **97**: 2109-2115 [PMID: 12190185 DOI: 10.1111/j.1572-0241.2002.05931.x]
53 **Muto T**, Kamiya J, Sawada T, Konishi F, Sugihara K, Kubota Y, Adachi M, Agawa S, Saito Y, Morioka Y. Small "flat adenoma" of the large bowel with special reference to its clinicopathologic features. *Dis Colon Rectum* 1985; **28**: 847-851 [PMID: 4053897]
54 **Soetikno R**, Friedland S, Kaltenbach T, Chayama K, Tanaka S. Nonpolypoid (flat and depressed) colorectal neoplasms. *Gastroenterology* 2006; **130**: 566-576; quiz 588-589 [PMID: 16472608 DOI: 10.1053/j.gastro.2005.12.006]
55 **Testoni PA**, Notaristefano C, Vailati C, Di Leo M, Viale E. High-definition colonoscopy with i-Scan: better diagnosis for small polyps and flat adenomas. *World J Gastroenterol* 2012; **18**: 5231-5239 [PMID: 23066318 DOI: 10.3748/wjg.v18.i37.5231]
56 **Morita T**, Tomita N, Ohue M, Sekimoto M, Yamamoto H, Ohnishi T, Tada M, Ikenaga M, Miyake Y, Sakita I, Tamaki Y, Matsuura N, Ito M, Monden M. Molecular analysis of diminutive, flat, depressed colorectal lesions: are they precursors of polypoid adenoma or early stage carcinoma? *Gastrointest Endosc* 2002; **56**: 663-671 [PMID: 12397273 DOI: 10.1067/mge.2002.129087]
57 **Saitoh Y**, Waxman I, West AB, Popnikolov NK, Gatalica Z, Watari J, Obara T, Kohgo Y, Pasricha PJ. Prevalence and distinctive biologic features of flat colorectal adenomas in a North American population. *Gastroenterology* 2001; **120**: 1657-1665 [PMID: 11375947]
58 **Yashiro M**, Carethers JM, Laghi L, Saito K, Slezak P, Jaramillo E, Rubio C, Koizumi K, Hirakawa K, Boland CR. Genetic pathways in the evolution of morphologically distinct colorectal neoplasms. *Cancer Res* 2001; **61**: 2676-2683 [PMID: 11289147]
59 **Kinney TP**, Merel N, Hart J, Joseph L, Waxman I. Microsatellite analysis of sporadic flat and depressed lesions of the colon. *Dig Dis Sci* 2005; **50**: 327-330 [PMID: 15745095]
60 **Rembacken BJ**, Fujii T, Cairns A, Dixon MF, Yoshida S, Chalmers DM, Axon AT. Flat and depressed colonic neoplasms: a prospective study of 1000 colonoscopies in the UK. *Lancet* 2000; **355**: 1211-1214 [PMID: 10770302]
61 **Hiraoka S**, Kato J, Tatsukawa M, Harada K, Fujita H, Morikawa T, Shiraha H, Shiratori Y. Laterally spreading type of colorectal adenoma exhibits a unique methylation phenotype and K-ras mutations. *Gastroenterology* 2006; **131**: 379-389 [PMID: 16890591 DOI: 10.1053/j.gastro.2006.04.027]
62 **Takayama T**, Katsuki S, Takahashi Y, Ohi M, Nojiri S, Sakamaki S, Kato J, Kogawa K, Miyake H, Niitsu Y. Aberrant crypt foci of the colon as precursors of adenoma and cancer. *N Engl J Med* 1998; **339**: 1277-1284 [PMID: 9791143 DOI: 10.1056/nejm199810293391803]
63 **Shpitz B**, Bomstein Y, Mekori Y, Cohen R, Kaufman Z, Neufeld D, Galkin M, Bernheim J. Aberrant crypt foci in human colons: distribution and histomorphologic characteristics. *Hum Pathol* 1998; **29**: 469-475 [PMID: 9596270]
64 **Adler DG**, Gostout CJ, Sorbi D, Burgart LJ, Wang L, Harmsen WS. Endoscopic identification and quantification

of aberrant crypt foci in the human colon. *Gastrointest Endosc* 2002; **56**: 657-662 [PMID: 12397272 DOI: 10.1067/mge.2002.128540]

65 **Hurlstone DP**, Karajeh M, Sanders DS, Drew SK, Cross SS. Rectal aberrant crypt foci identified using high-magnification-chromoscopic colonoscopy: biomarkers for flat and depressed neoplasia. *Am J Gastroenterol* 2005; **100**: 1283-1289 [PMID: 15929758 DOI: 10.1111/j.1572-0241.2005.40891.x]

66 **Kim J**, Ng J, Arozulllah A, Ewing R, Llor X, Carroll RE, Benya RV. Aberrant crypt focus size predicts distal polyp histopathology. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 1155-1162 [PMID: 18483337 DOI: 10.1158/1055-9965.epi-07-2731]

67 **Moxon D**, Raza M, Kenney R, Ewing R, Arozullah A, Mason JB, Carroll RE. Relationship of aging and tobacco use with the development of aberrant crypt foci in a predominantly African-American population. *Clin Gastroenterol Hepatol* 2005; **3**: 271-278 [PMID: 15765447]

68 **Rudolph RE**, Dominitz JA, Lampe JW, Levy L, Qu P, Li SS, Lampe PD, Bronner MP, Potter JD. Risk factors for colorectal cancer in relation to number and size of aberrant crypt foci in humans. *Cancer Epidemiol Biomarkers Prev* 2005; **14**: 605-608 [PMID: 15767337 DOI: 10.1158/1055-9965.epi-04-0058]

69 **Sakai E**, Takahashi H, Kato S, Uchiyama T, Hosono K, Endo H, Maeda S, Yoneda M, Taguri M, Nakajima A. Investigation of the prevalence and number of aberrant crypt foci associated with human colorectal neoplasm. *Cancer Epidemiol Biomarkers Prev* 2011; **20**: 1918-1924 [PMID: 21750169 DOI: 10.1158/1055-9965.epi-11-0104]

70 **Seike K**, Koda K, Oda K, Kosugi C, Shimizu K, Nishimura M, Shioiri M, Takano S, Ishikura H, Miyazaki M. Assessment of rectal aberrant crypt foci by standard chromoscopy and its predictive value for colonic advanced neoplasms. *Am J Gastroenterol* 2006; **101**: 1362-1369 [PMID: 16771962 DOI: 10.1111/j.1572-0241.2006.00578.x]

71 **Stevens RG**, Swede H, Heinen CD, Jablonski M, Grupka M, Ross B, Parente M, Tirnauer JS, Giardina C, Rajan TV, Rosenberg DW, Levine J. Aberrant crypt foci in patients with a positive family history of sporadic colorectal cancer. *Cancer Lett* 2007; **248**: 262-268 [PMID: 16950561 DOI: 10.1016/j.canlet.2006.08.003]

72 **Uchiyama T**, Takahashi H, Endo H, Kato S, Sakai E, Hosono K, Yoneda M, Inamori M, Hippo Y, Nakagama H, Nakajima A. Number of aberrant crypt foci in the rectum is a useful surrogate marker of colorectal adenoma recurrence. *Dig Endosc* 2012; **24**: 353-357 [PMID: 22925289 DOI: 10.1111/j.1443-1661.2012.01289.x]

73 **Cho NL**, Redston M, Zauber AG, Carothers AM, Hornick J, Wilton A, Sontag S, Nishioka N, Giardiello FM, Saltzman JR, Gostout C, Eagle CJ, Hawk ET, Bertagnolli MM. Aberrant crypt foci in the adenoma prevention with celecoxib trial. *Cancer Prev Res* (Phila) 2008; **1**: 21-31 [PMID: 19138933 DOI: 10.1158/1940-6207.capr-07-0011]

74 **Higurashi T**, Hosono K, Endo H, Takahashi H, Iida H, Uchiyama T, Ezuka A, Uchiyama S, Yamada E, Ohkubo H, Sakai E, Maeda S, Morita S, Natsumeda Y, Nagase H, Nakajima A. Eicosapentaenoic acid (EPA) efficacy for colorectal aberrant crypt foci (ACF): a double-blind randomized controlled trial. *BMC Cancer* 2012; **12**: 413 [PMID: 22992267 DOI: 10.1186/1471-2407-12-413]

75 **Higurashi T**, Takahashi H, Endo H, Hosono K, Yamada E, Ohkubo H, Sakai E, Uchiyama T, Hata Y, Fujisawa N, Uchiyama S, Ezuka A, Nagase H, Kessoku T, Matsuhashi N, Yamanaka S, Inayama Y, Morita S, Nakajima A. Metformin efficacy and safety for colorectal polyps: a double-blind randomized controlled trial. *BMC Cancer* 2012; **12**: 118 [PMID: 22443173 DOI: 10.1186/1471-2407-12-118]

76 **Hosono K**, Endo H, Takahashi H, Sugiyama M, Sakai E, Uchiyama T, Suzuki K, Iida H, Sakamoto Y, Yoneda K, Koide T, Tokoro C, Abe Y, Inamori M, Nakagama H, Nakajima A. Metformin suppresses colorectal aberrant crypt foci in a short-term clinical trial. *Cancer Prev Res* (Phila) 2010; **3**: 1077-1083 [PMID: 20810669 DOI: 10.1158/1940-6207.capr-10-0186]

77 **Takayama T**, Nagashima H, Maeda M, Nojiri S, Hirayama M, Nakano Y, Takahashi Y, Sato Y, Sekikawa H, Mori M, Sonoda T, Kimura T, Kato J, Niitsu Y. Randomized double-blind trial of sulindac and etodolac to eradicate aberrant crypt foci and to prevent sporadic colorectal polyps. *Clin Cancer Res* 2011; **17**: 3803-3811 [PMID: 21385928 DOI: 10.1158/1078-0432.ccr-10-2395]

78 **Sakai E**, Morioka T, Yamada E, Ohkubo H, Higurashi T, Hosono K, Endo H, Takahashi H, Takamatsu R, Cui C, Shiozawa M, Akaike M, Samura H, Nishimaki T, Nakajima A, Yoshimi N. Identification of preneoplastic lesions as mucin-depleted foci in patients with sporadic colorectal cancer. *Cancer Sci* 2012; **103**: 144-149 [PMID: 22017323 DOI: 10.1111/j.1349-7006.2011.02125.x]

79 **Takayama T**, Ohi M, Hayashi T, Miyanishi K, Nobuoka A, Nakajima T, Satoh T, Takimoto R, Kato J, Sakamaki S, Niitsu Y. Analysis of K-ras, APC, and beta-catenin in aberrant crypt foci in sporadic adenoma, cancer, and familial adenomatous polyposis. *Gastroenterology* 2001; **121**: 599-611 [PMID: 11522744]

80 **Chan AO**, Broaddus RR, Houlihan PS, Issa JP, Hamilton SR, Rashid A. CpG island methylation in aberrant crypt foci of the colorectum. *Am J Pathol* 2002; **160**: 1823-1830 [PMID: 12000733 DOI: 10.1016/s0002-9440(10)61128-5]

81 **Otori K**, Konishi M, Sugiyama K, Hasebe T, Shimoda T, Kikuchi-Yanoshita R, Mukai K, Fukushima S, Miyaki M, Esumi H. Infrequent somatic mutation of the adenomatous polyposis coli gene in aberrant crypt foci of human colon tissue. *Cancer* 1998; **83**: 896-900 [PMID: 9731892]

82 **Lao VV**, Grady WM. Epigenetics and colorectal cancer. *Nat Rev Gastroenterol Hepatol* 2011; **8**: 686-700 [PMID: 22009203 DOI: 10.1038/nrgastro.2011.173]

83 **Rosenberg DW**, Yang S, Pleau DC, Greenspan EJ, Stevens RG, Rajan TV, Heinen CD, Levine J, Zhou Y, O'Brien MJ. Mutations in BRAF and KRAS differentially distinguish serrated versus non-serrated hyperplastic aberrant crypt foci in humans. *Cancer Res* 2007; **67**: 3551-3554 [PMID: 17440063 DOI: 10.1158/0008-5472.can-07-0343]

84 **Ahuja N**, Issa JP. Aging, methylation and cancer. *Histol Histopathol* 2000; **15**: 835-842 [PMID: 10963127]

85 **Issa JP**, Ottaviano YL, Celano P, Hamilton SR, Davidson NE, Baylin SB. Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. *Nat Genet* 1994; **7**: 536-540 [PMID: 7951326 DOI: 10.1038/ng0894-536]

86 **Toyota M**, Issa JP. CpG island methylator phenotypes in aging and cancer. *Semin Cancer Biol* 1999; **9**: 349-357 [PMID: 10547343 DOI: 10.1006/scbi.1999.0135]

87 **Slaughter DP**, Southwick HW, Smejkal W. Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer* 1953; **6**: 963-968 [PMID: 13094644]

88 **Suzuki H**, Watkins DN, Jair KW, Schuebel KE, Markowitz SD, Chen WD, Pretlow TP, Yang B, Akiyama Y, Van Engeland M, Toyota M, Tokino T, Hinoda Y, Imai K, Herman JG, Baylin SB. Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. *Nat Genet* 2004; **36**: 417-422 [PMID: 15034581 DOI: 10.1038/ng1330]

89 **Kawakami K**, Ruszkiewicz A, Bennett G, Moore J, Grieu F, Watanabe G, Iacopetta B. DNA hypermethylation in the normal colonic mucosa of patients with colorectal cancer. *Br J Cancer* 2006; **94**: 593-598 [PMID: 16421593 DOI: 10.1038/sj.bjc.6602940]

90 **Belshaw NJ**, Elliott GO, Foxall RJ, Dainty JR, Pal N, Coupe A, Garg D, Bradburn DM, Mathers JC, Johnson IT. Profiling CpG island field methylation in both morphologically normal and neoplastic human colonic mucosa. *Br J Cancer* 2008;

99: 136-142 [PMID: 18542073 DOI: 10.1038/sj.bjc.6604432]
91 **Hiraoka S**, Kato J, Horii J, Saito S, Harada K, Fujita H, Kuriyama M, Takemoto K, Uraoka T, Yamamoto K. Methylation status of normal background mucosa is correlated with occurrence and development of neoplasia in the distal colon. *Hum Pathol* 2010; **41**: 38-47 [PMID: 19733896 DOI: 10.1016/j.humpath.2009.06.002]
92 **Silviera ML**, Smith BP, Powell J, Sapienza C. Epigenetic differences in normal colon mucosa of cancer patients suggest altered dietary metabolic pathways. *Cancer Prev Res* (Phila) 2012; **5**: 374-384 [PMID: 22300984 DOI: 10.1158/1940-6207.capr-11-0336]
93 **Hewitson P**, Glasziou P, Watson E, Towler B, Irwig L. Cochrane systematic review of colorectal cancer screening using the fecal occult blood test (hemoccult): an update. *Am J Gastroenterol* 2008; **103**: 1541-1549 [PMID: 18479499 DOI: 10.1111/j.1572-0241.2008.01875.x]
94 **Mori Y**, Olaru AV, Cheng Y, Agarwal R, Yang J, Luvsanjav D, Yu W, Selaru FM, Hutfless S, Lazarev M, Kwon JH, Brant SR, Marohn MR, Hutcheon DF, Duncan MD, Goel A, Meltzer SJ. Novel candidate colorectal cancer biomarkers identified by methylation microarray-based scanning. *Endocr Relat Cancer* 2011; **18**: 465-478 [PMID: 21636702 DOI: 10.1530/erc-11-0083]
95 **Simmer F**, Brinkman AB, Assenov Y, Matarese F, Kaan A, Sabatino L, Villanueva A, Huertas D, Esteller M, Lengauer T, Bock C, Colantuoni V, Altucci L, Stunnenberg HG. Comparative genome-wide DNA methylation analysis of colorectal tumor and matched normal tissues. *Epigenetics* 2012; **7**: 1355-1367 [PMID: 23079744 DOI: 10.4161/epi.22562]
96 **Yi JM**, Dhir M, Guzzetta AA, Iacobuzio-Donahue CA, Heo K, Yang KM, Suzuki H, Toyota M, Kim HM, Ahuja N. DNA methylation biomarker candidates for early detection of colon cancer. *Tumour Biol* 2012; **33**: 363-372 [PMID: 22238052 DOI: 10.1007/s13277-011-0302-2]
97 **Ahlquist DA**, Zou H, Domanico M, Mahoney DW, Yab TC, Taylor WR, Butz ML, Thibodeau SN, Rabeneck L, Paszat LF, Kinzler KW, Vogelstein B, Bjerregaard NC, Laurberg S, Sørensen HT, Berger BM, Lidgard GP. Next-generation stool DNA test accurately detects colorectal cancer and large adenomas. *Gastroenterology* 2012; **142**: 248-56; quiz e25-6 [PMID: 22062357 DOI: 10.1053/j.gastro.2011.10.031]
98 **Tóth K**, Sipos F, Kalmár A, Patai AV, Wichmann B, Stoehr R, Golcher H, Schellerer V, Tulassay Z, Molnár B. Detection of methylated SEPT9 in plasma is a reliable screening method for both left- and right-sided colon cancers. *PLoS One* 2012; **7**: e46000 [PMID: 23049919 DOI: 10.1371/journal.pone.0046000]
99 **Popat S**, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. *J Clin Oncol* 2005; **23**: 609-618 [PMID: 15659508 DOI: 10.1200/jco.2005.01.086]
100 **Maughan TS**, Adams RA, Smith CG, Meade AM, Seymour MT, Wilson RH, Idziaszczyk S, Harris R, Fisher D, Kenny SL, Kay E, Mitchell JK, Madi A, Jasani B, James MD, Bridgewater J, Kennedy MJ, Claes B, Lambrechts D, Kaplan R, Cheadle JP. Addition of cetuximab to oxaliplatin-based first-line combination chemotherapy for treatment of advanced colorectal cancer: results of the randomised phase 3 MRC COIN trial. *Lancet* 2011; **377**: 2103-2114 [PMID: 21641636 DOI: 10.1016/s0140-6736(11)60613-2]
101 **Allegra CJ**, Jessup JM, Somerfield MR, Hamilton SR, Hammond EH, Hayes DF, McAllister PK, Morton RF, Schilsky RL. American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. *J Clin Oncol* 2009; **27**: 2091-2096 [PMID: 19188670]

P- Reviewers: Lakatos PL, Milone M, Sipos F **S- Editor**: Gou SX
L- Editor: A **E- Editor**: Ma S

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.988

World J Gastroenterol 2014 January 28; 20(4): 988-996
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Radiofrequency ablation as treatment for pulmonary metastasis of colorectal cancer

Takao Hiraki, Hideo Gobara, Toshihiro Iguchi, Hiroyasu Fujiwara, Yusuke Matsui, Susumu Kanazawa

Takao Hiraki, Hideo Gobara, Toshihiro Iguchi, Hiroyasu Fujiwara, Yusuke Matsui, Susumu Kanazawa, Department of Radiology, Okayama University Medical School, Okayama 700-8558, Japan
Author contributions: Hiraki T contributed to literature review, manuscript writing, and final revision of the article; Gobara H, Iguchi T, Fujiwara H, Matsui Y and Kanazawa S contributed to literature review, significant revisions to drafts of the article, and approval of the final revision of the article.
Correspondence to: Takao Hiraki, MD, Department of Radiology, Okayama University Medical School, 2-5-1 Shikatacho, Okayama 700-8558, Japan. takaoh@tc4.so-net.ne.jp
Telephone: +81-86-2357313 Fax: +81-86-2357316
Received: September 17, 2013 Revised: November 29, 2013
Accepted: January 6, 2014
Published online: January 28, 2014

Abstract

Radiofrequency ablation (RFA) causes focal coagulation necrosis in tissue. Its first clinical application was reported in 2000, and RFA has since been commonly used in both primary and metastatic lung cancer. The procedure is typically performed using computed tomography guidance, and the techniques for introducing the electrode to the tumor are simple and resemble those used in percutaneous lung biopsy. The most common complication is pneumothorax, which occurs in up to 50% of procedures; chest tube placement for pneumothorax is required in up to 25% of procedures. Other severe complications, such as pleural effusion requiring chest tube placement, infection, and nerve injury, are rare. The local efficacy depends on tumor size, and local progression after RFA is not rare, occurring in 10% or more of patients. The local progression rate is particularly high for tumors > 3 cm. Repeat RFA may be used to treat local progression. Short- to mid-term survival after RFA appears promising and is approximately 85%-95% at 1 year and 45%-55% at 3 years. Long-term survival data are sparse. Better survival may be expected for patients with small metastasis, low carcinoembryonic antigen levels, and/or no extrapulmonary metastasis. The notable advantages of RFA are that it is simple and minimally invasive; preserves pulmonary function; can be repeated; and is applicable regardless of previous treatments. Its most substantial limitation is limited local efficacy. Although surgery is still the method of choice for treatment with curative intent, the ultimate application of RFA may be to replace metastasectomy for small metastases. Randomized trials comparing RFA with surgery are needed.

Key words: Radiofrequency ablation; Pulmonary metastases; Colorectal cancer; Ablation therapy; Lung

Core tip: Radiofrequency ablation (RFA) for pulmonary metastasis of colorectal cancer is technically simple. The procedure rarely results in death. The most common complication is pneumothorax, which occurs in up to 50% of patients. Severe complications are rare. Local progression after RFA is not rare and occurs in 10% or more of cases. The short- to mid-term survival after RFA appears promising and is approximately 85%-95% at 1 year and 45%-55% at 3 years. Long-term survival data are sparse. Better survival may be expected for patients with small metastasis, low carcinoembryonic antigen levels, and/or no extrapulmonary metastasis.

Hiraki T, Gobara H, Iguchi T, Fujiwara H, Matsui Y, Kanazawa S. Radiofrequency ablation as treatment for pulmonary metastasis of colorectal cancer. *World J Gastroenterol* 2014; 20(4): 988-996 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/988.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.988

INTRODUCTION

Colon cancer is the third most common cancer and the second most common cause of cancer-related mortality in the United States, and 10%-30% of patients with colon cancer have pulmonary metastasis at presentation[1,2]. Even if metastasis is not initially present, the cancer may recur in the lungs after curative resection of the primary cancer. Kobayashi *et al*[3] surveyed 5230 patients who underwent curative resection for colorectal cancer and found that 906 patients (17%) developed recurrence at a median of 1.4 years after surgery. The first recurrence site in 250 patients (5%) was the lungs, which was the second most common site of recurrence after the liver (373 patients, 7%). Although lung recurrence is usually accompanied by recurrence at other sites, recurrence was confined to the lungs in 2%-10% of patients who develop distant metastases[4,5].

A meta-analysis demonstrated that patients with untreated locally advanced or metastatic colorectal cancer had a median survival of 8 mo[6]. The International Registry of Lung Metastases[7] revealed that the 5-year survival rate for patients who underwent complete resection of lung metastasis was 36%, compared to 13% for patients who did not undergo complete resection. A large-scale, multicenter retrospective study in Japan[3] also reported significantly better survival in patients who underwent resection for pulmonary recurrence. Thus, surgery is considered the treatment of choice for curative intent. However, that study[3] also indicated that less than half (38%) of the patients with pulmonary recurrence underwent surgical resection. Mitry *et al*[2] reported that only 4% of patients with synchronous pulmonary metastases and 14% of patients with metachronous pulmonary metastases were curatively resected. These data indicate that many patients with pulmonary metastases are not considered suitable for surgery. Therefore, the development of less invasive local therapies, such as radiofrequency ablation (RFA), may be attractive.

PRINCIPLE AND TECHNIQUES OF LUNG RFA

RFA causes focal coagulation necrosis in tissue via the delivery of energy in the form of an alternating electrical current with a frequency of 460-500 kHz in the radio wave range. The alternating electrical current causes the agitation of ionic dipolar molecules in surrounding tissue and fluids, resulting in frictional heating. The exposure of cells to temperatures of 50-52 ℃ for 4-6 min may induce cytotoxicity[8]. Between 60 ℃ and 100 ℃, there is a near instantaneous induction of protein coagulation, which irreversibly damages key cytosolic and mitochondrial enzymes, as well as nucleic acid-histone protein complexes[8]. Thus, the aim of the RFA procedure is to generate temperatures > 50 ℃ in cancer cells.

Since Dupuy *et al*[9] reported the first clinical use of RFA to treat lung cancer in 2000, RFA has been commonly used as a treatment for both primary and metastatic lung cancer. The thermal and electrical conductivity of air are low, and thus the effects of RFA on the lungs may be tissue-specific. Accordingly, it has been demonstrated that a given quantity of RF current produces a larger volume of ablation of tumors in the lungs than in subcutaneous tissues or the kidneys[10]. Conversely, alveolar air and ventilation may limit the ablation zone in the surrounding parenchyma, as saline infusion into the lung parenchyma to reduce alveolar air and bronchial balloon occlusion enlarged the ablation zone in animal experiments[11,12]. This difficulty in ablating the marginal parenchyma may account for the relatively high frequency of local progression after RFA of lung cancer.

RFA is indicated in patients who are considered non-surgical candidates and for whom the treatment of lung cancer is expected to contribute to prolonged survival. The procedure is not indicated in patients with poor performance status (*e.g.*, PS $\geqslant$ 3), leucocyte count < 3000 cells/μL, uncorrected coagulopathy (*e.g.*, a platelet count < 50000/μL or a prothrombin time-international ratio > 1.5), poor pulmonary function (*e.g.*, predicted forced respiratory volume in 1 sec $\leqslant$ 1000 mL), poor cardiac function (*e.g.*, New York Heart Association Class $\geqslant$ III), uncorrected diabetes (*e.g.*, HbA1c $\geqslant$ 7), and uncontrollable extrapulmonary cancer. The procedure is feasible, but patients with tumors in contact with the heart and aorta are at a higher risk of local progression[13].

The electrode used for lung RFA is usually either a multitined expandable electrode or an internally cooled electrode[14]. The multitined expandable electrode, which is more commonly used for lung RFA, consists of an array of multiple electrode tines that expand from a single, centrally positioned large needle cannula. The internally cooled electrode consists of dual-lumen needles with non-insulated active tips, in which internal cooling is achieved by continuous perfusion with chilled saline.

We suggest that the procedure should be performed by physicians who are familiar with both computed tomography (CT)-guided intervention and RFA. The procedure is usually conducted under local anesthesia, but epidural or general anesthesia may also be used. CT is the only image-guidance modality that can be used for lung RFA. CT fluoroscopy permits a near real-time image display, thereby facilitating the procedure. The techniques used to introduce the electrode into the tumor under CT guidance are simple and similar to those used in percutaneous lung biopsy. A prospective multicenter clinical trial showed that treatment was successfully completed in 99% (105/106) of patients[15]. Multiplanar reconstruction of CT images is useful for confirming proper positioning of the electrode. After introducing the electrode into the tumor, a given RF energy is applied for a variable duration. In our institution, an ablation algorithm based on electrode type is used; this algorithm has been described in the literature[16]. The procedure should aim to obtain an ablative margin of at least 0.5 cm around the tumor to treat the microscopic extension of cancer cells around

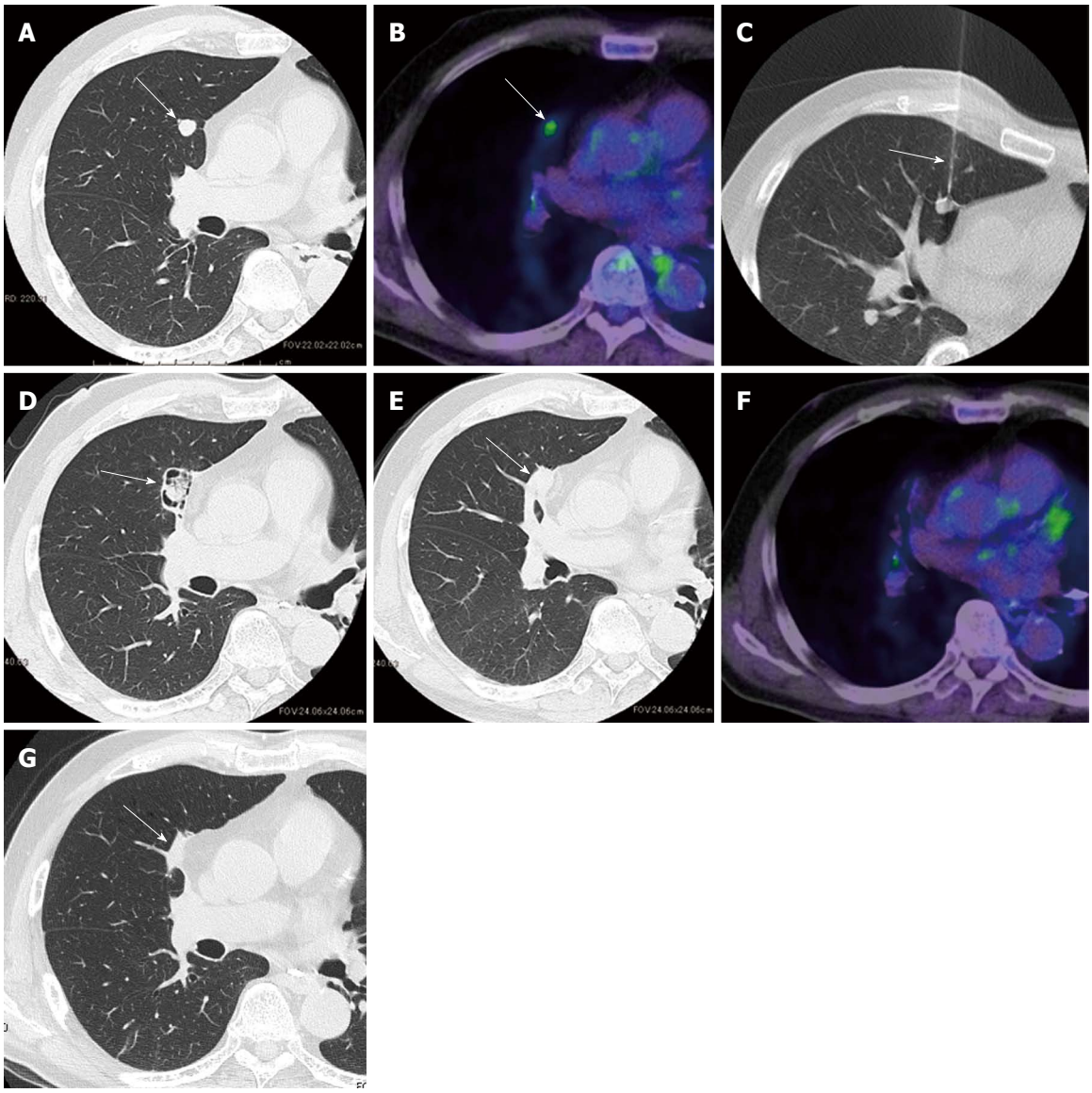


Figure 1 Pulmonary metastasis in a 68-year-old man with colorectal cancer treated with radiofrequency ablation. A: Computer tomography (CT) image before radiofrequency ablation (RFA) showing a tumor (arrow) 1.1 cm in size in the right middle lobe; B: Positron emission tomography (PET) image before RFA showing increased fluorodeoxyglucose (FDG) uptake by the tumor (arrow); C: CT fluoroscopic image obtained during RFA showing the treatment of the tumor with a multitined expandable electrode (arrow); D: CT image 1 mo after RFA showing cavity formation around the ablated tumor (arrow); E: CT image 3 mo after RFA showing cavity collapse and an increase in the size of the ablation zone (arrow) beyond the tumor size before RFA; F: PET image 6 mo after RFA showing the disappearance of FDG uptake; G: CT image 24 mo after RFA showing the shrinkage of the ablation zone (arrow) and its appearance as a focal atelectasis.

the macroscopic mass and thereby decrease the risk of local progression. To obtain an adequate ablative margin, repositioning of the electrode followed by application of RF energy (so-called "multiple overlapping ablations") may be performed.

RADIOLOGICAL EVALUATION OF LOCAL EFFICACY

Figure 1 shows radiological images of a pulmonary metastasis from a colorectal cancer patient treated with RFA. Local efficacy is evaluated primarily by sequential follow-up CT scans. During the first 6 mo after RFA, the size of the ablated lesion may exceed the tumor size before ablation because the lesion includes the ablated marginal parenchyma surrounding the tumor[17-19]. Thus, at a given time point during this period, local efficacy cannot be evaluated by comparing the tumor size with the pretreatment tumor size. Consequently, CT images are first obtained in the early period (*e.g.*, 1 mo) after RFA as a point of reference. Thereafter, it is possible to evaluate local efficacy by comparing the size and geometry of the ablation zone with the previous CT images. When the tumor is completely ablated, the ablation zone gradually decreases in size[20] and typically becomes scar-like tissue. Local tumor progression is considered to occur when the ablation zone increases in size[20]. In our experience, in most cases of local tumor progression, a nodule appears in the periphery of the ablation zone that always enlarges

if untreated. Such a nodule generally exhibits some degree of contrast enhancement that distinguishes it from the unenhanced necrotic tumor tissue[20]. Thus, contrast-enhanced CT images can be helpful in confirming the diagnosis of local progression. However, in our experience, local progression is diagnosed by careful observation of the size and geometry of the ablation zones. Therefore, we are of the opinion that contrast-enhanced CT is preferable but not essential for diagnosing local progression.

Positron emission tomography may also be used to evaluate local efficacy. Focal areas of increased fluorodeoxyglucose uptake at the ablated zone are suggestive of local tumor progression. However, attention should be paid to possible false-positive results during the first 3 mo[17,21] or even at 24 mo[22] after RFA, due to inflammation induced by RFA.

REVIEW OF STUDIES ON RFA OF PULMONARY METASTASES FROM COLORECTAL CANCER

A review of the literature was conducted by searching the PubMed database. The results were limited to studies published in English, and the search was performed with the keywords "colorectal", "lung", and "radiofrequency ablation". The citations of all electronically identified articles were further manually searched for potentially relevant studies. Human clinical studies on the efficacy of RFA of pulmonary metastases from colorectal cancer were selected, while animal experiments, case reports, and reviews were excluded. All relevant articles were subsequently evaluated.

Table 1 summarizes the results for the use of RFA to treat pulmonary metastases in patients with colorectal cancer. A group at St. George Hospital in Australia published several reports on the use of RFA to treat pulmonary metastases in patients with colorectal cancer[18,23-28]. In 2003, Steinke *et al*[18] published their preliminary study, which mainly focused on morbidity. In total, 20 nonsurgical candidates with 41 pulmonary metastases from colorectal cancer were treated with RFA. The procedure resulted in technical failure for 1 tumor. A total of 10 (50%) patients developed pneumothorax, and 5 patients (25%) required chest tube placement. Intrapulmonary hemorrhage occurred in 3 (7.5%) of the 40 tumors, but all cases were self-limiting. In 2007, Yan *et al*[26] reported the mid-term outcomes of 55 nonsurgical candidates, including morbidities, local efficacy, and survival. No hospital mortality was reported. The periprocedural morbidity rate was 42%, which included intrapulmonary bleeding (9%), pneumothorax (29%), pleural effusion (7%), and persistent pleuritic chest pain for more than 1 wk (4%). Some patients experienced more than 1 adverse event. In total, 9 patients had pneumothorax that required chest tube placement (16%). The median duration of hospital stay was 1 d, and the median follow-up period was 24 mo. The proportion of local progression at the time of the study was 38%. The 1-, 2-, and 3-year overall survival rates were 85%, 64%, and 46%, respectively, and the median overall survival was 33 mo. The 1- and 2-year local progression-free survival rates were 74% and 56%, respectively. The 1- and 2-year local progression-free survival rates were 88% and 69%, respectively, for the patients in which the largest lung metastasis was $\leqslant$ 3 cm, and 27% and 18%, respectively, for the patients in which the largest lung metastasis was > 3 cm. The 1- and 2-year overall progression-free survival rates were 61% and 34%, respectively. The median overall progression-free survival was 15 mo. Univariate analyses identified the following factors as significant for local progression-free survival: the size of the largest lung metastasis, the location of the lung metastases, and post-RFA carcinoembryonic antigen (CEA) levels at 1 and 3 mo. According to multivariate analysis, a largest lung metastasis of > 3 cm (HR = 8.3) and post-RFA CEA level of > 5 ng/mL at 1 mo (HR = 3.5) were independently associated with reduced local progression-free survival. Two factors were found to be significant for overall progression-free survival: sex and size of the largest lung metastasis. In multivariate analysis, only a largest lung metastasis of > 3 cm (HR = 5.1) was independently associated with reduced overall progression-free survival. Yan *et al*[27] also reported a learning curve for RFA in which morbidity was reduced. The same group[28] reported the outcomes of an open-label prospective trial of RFA for 148 nonsurgical candidates with lung metastases from several primary cancers; 73% of these patients had primary colorectal cancer. Although the data for the colorectal cancer patient subgroup was limited, the median overall survival for patients with colorectal cancer was found to be 60 mo.

Simon *et al*[29] reported a mixed population comprising 153 nonsurgical candidates with 189 lung cancers, including 18 patients with pulmonary metastasis from colorectal cancer. Although the data from the colorectal cancer subgroup were scarce, the 1-, 2-, 3-, and 5-year survival rates for those patients were 87%, 78%, 57%, and 57%, respectively. Lencioni *et al*[15] performed a prospective, multicenter clinical trial of RFA using a mixed population comprising 106 nonsurgical candidates with primary lung cancer and pulmonary metastasis from various primary cancers. In total, 53 patients had metastases from colorectal cancer. No procedure-related deaths occurred. Complete treatment was confirmed for $\geqslant$ 1 year in 91% of the patients with pulmonary metastases from colorectal cancer. The 1- and 2-year overall survival rates for the patients with colorectal metastases were 89% and 66%, respectively. The cancer-specific 1- and 2-year survival rates were 91% and 68%, respectively.

Hiraki *et al*[30] assessed the outcomes of 27 nonsurgical candidates with pulmonary metastases from colorectal cancer; these patients comprised a total of 41 RFA sessions. There was no mortality or sequela. Pneumothorax occurred after 49% of the sessions, and chest tube placement was required after 7.3% of the sessions. Pleural

Table 1 Summary of studies reporting outcomes of radiofrequency ablation of pulmonary metastases from colorectal cancer

Ref.	Center	Year	*n*	Patient age (yr)	No. of tumors per patient	Tumor size (cm)	Follow-up period (mo)	Mortality and morbidity	Local efficacy	Survival	Prognostic factors
Steinke *et al*[18]	St. George Hospital	2003	20	62 (mean)	2.1	1.4 (mean)	14 (median)	Mortality: 0%, Overall PTX: 50%, PTX requiring chest tube placement: 25%, Self-limiting intrapulmonary hemorrhage: 7.5%	NA	NA	NA
Yan *et al*[26]	St. George Hospital	2007	55	62 (mean)	NA	2.1 (mean)	24 (median)	Mortality: 0%, Overall morbidity rate: 42%, PTX: 29%, Pleural effusion: 7%, PTX requiring chest tube placement: 16%, Self-limiting intrapulmonary hemorrhage: 9%	Proportion of local tumor progression: 38%	1-/2-/3-year OS rate: 85%/64%/46%, respectively, Median OS: 33 mo, 1-/2-year local PFS rate: 74%/56%, respectively, 1-/2-year overall PFS rate: 61%/34%, respectively, Median overall PFS: 15 mo	Tumor size and CEA level at 1 month after RFA for local PFS by multivariate analyses, Tumor size for overall PFS by multivariate analyses
Lencioni *et al*[15]	Multicenter in the United States, United kingdom, Italy, Germany, and Australia	2008	53	63 (mean)	2.2	1.4 (mean)	NA	Mortality: 0%	Proportion of local tumor progression: 9%	OS 1-/2-year: 89%/66%, Cancer-specific survival 1-/2-year: 91%/68%	NA
Hiraki *et al*[30]	Okayama University	2007	27	62 (mean)	1.8	1.5 (mean)	20 (median)	Mortality: 0%, Overall PTX: 49%, PTX requiring chest tube placement: 7.3%, Pleural effusion: 15%,	Primary and secondary proportion of local tumor progression: 31% and 20%, respectively 1-/2-/3-year primary and secondary local control rate: 72% and 85%/56% and 62%/56% and 62%, respectively	1-/2-/3-year OS rate: 96%/54%/48%, respectively, Mean OS: 33 mo	Extrapulmonary metastasis for OS by univariate analyses
Yamakado *et al*[31]	Multicenter in Japan	2007	71	64 (mean)	2.2	2.4 (mean)	19 (mean)	Mortality: 0%, Overall PTX: 37%, PTX requiring chest tube placement: 20%, Pleural effusion: 14%, > 38 ℃ fever: 20%, Empyema requiring chest tube placement: 1.4%	Proportion of local tumor progression: 17%	1-/2-/3-year OS rate: 84%/62%/46%, respectively, Median OS: 31 mo	Extrapulmonary metastasis and tumor size for OS by multivariate analyses
Yamakado *et al*[32]	Mie University	2009	78	66 (mean)	2.5	2.0 (mean)	25 (mean)	Mortality: 0%, Overall PTX: 22%, PTX requiring chest tube placement: 13%, Pleural effusion requiring chest tube placement: 1.4%	Proportion of local tumor progression: 14%, 1-/3-/5-year local control rate: 90%/79%/79%	1-/3-/5-year OS rate: 84%/56%/35%, respectively, Median OS: 38 mo	Extrapulmonary metastasis and CEA level for OS by multivariate analyses
Petre *et al*[33]	Memorial Sloan-Kettering Cancer Center	2013	45	63 (mean)	1.5	0.4–3.5	18 (median)	Mortality: 0%, Overall PTX: 33%, PTX requiring chest tube placement: 19%, Overall pleural effusion: 5%, Pleural effusion requiring chest tube placement: 1.7%, Pneumonia: 1.7%	Primary and secondary proportion of local tumor progression: 13% and 7.2%, respectively	1-/2-/3-year OS rate: 95%/72%/50%, respectively, Median OS: 46 mo, 1-/2-/3-year primary and secondary local PFS rate: 92% and 95%/77% and 89%/77% and 89%, respectively	Number of pulmonary metastasis for OS by univariate analyses

Gillams *et al*[34]	University College London Medical School	2013	122	68 (median)	3.3	1.7 (mean)	NA	Mortality: 0%, Late procedure-related death: 0.4%, Major complication: 3.9%, PTX requiring chest tube placement: 15%, Pleural effusion requiring chest tube placement: 1.2%, Infection: 2.0%, Nerve injury: 0.8%	Proportion of local tumor progression: 19%	OS 3-year rate: 57%, Median OS: 41 mo	None

NA: Not available; PTX: Pneumothorax; OS: Overall survival; PFS: Progression-free survival; CEA: Carcinoembryonic antigen; RFA: Radiofrequency ablation.

effusion was encountered after 15% of the sessions. Local progression after RFA was observed in 31% (15/49) of the tumors; 5 of these locally progressing tumors were completely treated by repeating the procedure. Thus, local progression was observed in 20% of the tumors at the time of study. The primary local control rates were 72% at 1 year, 56% at 2 years, and 56% at 3 years. By repeating the procedure for local progression, the local control rates were improved to 85% at 1 year, 62% at 2 years, and 62% at 3 years. The 1-, 2-, and 3-year survival rates were 96%, 54%, and 48%, respectively. The mean survival time was 33 mo. Univariate analysis revealed that the presence of extrapulmonary metastasis at the time of RFA was the only significant factor associated with survival.

Yamakado *et al*[31] reported the results of a multicenter study in Japan comprising 71 nonsurgical candidates. No mortality was observed. Fever (> 38 ℃) developed in 14 patients (20%), and asymptomatic pleural effusion was observed in 10 (14%) patients. Pneumothorax developed in 26 (37%) patients, 14 (20%) of whom required a chest tube. Empyema developed in 1 (1.4%) patient. Local tumor progression was observed in 12 (17%) of the 71 patients during the mean follow-up period of 19 mo. The proportion of patients with local tumor progression was 11% (7/61) in those with tumors ≤ 3 cm and 50% (5/10) in those with tumors > 3 cm. This difference was statistically significant. The 1-, 2-, and 3-year overall survival rates were 84%, 62%, and 46%, respectively. The median survival time was 31 mo. Univariate analyses revealed that extrapulmonary metastasis, tumor size, and CEA level were significant prognostic factors. The first 2 factors were also significant according to multivariate analysis. Subsequently, Yamakado *et al*[32] reported a single-center study involving 78 patients with pulmonary metastases from colorectal cancer. The mean follow-up period was 24.6 mo. Pneumothorax developed in 22% (31/140) of the sessions, and pneumothorax and pleural effusion requiring chest tube placement occurred in 13% (18/140) and 1.4% (2/140) of the sessions, respectively. Local tumor progression was observed in 11 patients (14%). The 1-, 3-, and 5-year local tumor progression rates were 10%, 21%, and 21%, respectively. The 1-, 3-, and 5-year local tumor progression rates were 5%, 14%, and 14% in patients with tumors ≤ 3 cm and 53%, 69%, and 69% in patients with tumors > 3 cm. This difference was statistically significant. The 1-, 3-, and 5-year survival rates were 84%, 56%, and 35%, respectively, and the median survival time was 38 mo. Univariate analyses identified maximum tumor diameter of ≤ 3 cm, single-lung metastasis, absence of extrapulmonary metastasis, and normal CEA levels as prognostic factors. Multivariate analysis also indicated that the latter 2 variables were significantly independent prognostic factors. The 1-, 3-, and 5-year survival rates were 98%, 83%, and 57%, respectively, in the 54 patients with no extrapulmonary metastases and 97%, 86%, and 63%, respectively, in the 33 patients with negative CEA levels.

Petre *et al*[33] studied 45 nonsurgical candidates with 69 pulmonary metastases (< 3.5 cm) from colorectal cancer. The median hospital stay was 1 d. There was no periprocedural mortality. Pneumothorax occurred in 33% of the sessions, with 12 (19%) patients requiring a percutaneous chest tube. There were 3 cases of pleural effusion, one of which required catheter drainage. One patient developed bacterial pneumonia. The median follow-up period was 18 mo after RFA. Of the 69 lesions, local tumor progression occurred in 9 lesions (13%) at a median of 11.1 mo after RFA. Lesions > 1.5 cm had a tendency toward a higher risk of local progression compared with lesions ≤ 1.5 cm (HR = 7.03). Among the lesions that progressed, 4 were re-treated with RFA, and the secondary (after repeat ablations) effectiveness rate was 93% (64/69 lesions). The primary and secondary local tumor progression-free survival rates were 92% and 95%, respectively, at 1 year, 77% and 89%, respectively, at 2 years, and 77% and 89%, respectively, at 3 years. The median overall survival time after the RFA procedure was 46 mo. The 1-, 2-, and 3-year overall survival rates from the time of RFA were 95%, 72%, and 50%, respectively. Univariate analyses using various variables revealed that the only significant prognostic factor was the number of pulmonary metastasis at the time of RFA.

Gillams *et al*[34] performed 256 RFA procedures in 122 patients with a total of 398 metastases. The major complication rate was 3.9%. There were no cases of prolonged air leak. The 30-d mortality rate was 0%. There were 10 major complications (3.9%): 3 pleural effusions requiring drain insertion; 5 infections, including 1 delayed infection that resulted in fatal hemoptysis; and 2 nerve injuries (1 recurrent laryngeal nerve injury and 1 brachial plexus injury). Pneumothorax requiring drainage occurred in 39 (15%) of the

procedures. The local progression analysis included 268 tumors with > 6 mo of imaging follow-up data available for review. On a tumor-by-tumor basis, 52 (19%) of 268 tumors progressed locally. The mean and median times to local progression were 9 and 8 mo (range 2-27 mo), respectively. The median overall survival and 3-year survival rate were 41 mo and 57%, respectively. No significant prognostic factors were identified, although survival tended to be better in patients with smaller tumors.

In summary, RFA for colorectal pulmonary metastasis is a safe procedure that rarely results in death. The most common complication is pneumothorax, which occurs in up to 50% of procedures. Chest tube placement for pneumothorax is required after up to 25% of procedures. Other severe complications, such as pleural effusion requiring chest tube placement, infection, and nerve injury, are rare. Local tumor progression after RFA is not rare (10% or more) and is particularly common for tumors > 3 cm. Short- to mid-term survival after RFA appears promising, with survival rates of approximately 85%-95% at 1 year and 45%-55% at 3 years. Long-term (5 years or more) survival data are sparse. Significant prognostic factors include number and size of pulmonary metastases, CEA levels, and extrapulmonary metastasis.

The ultimate application of RFA may be to replace metastasectomy. Accordingly, survival data after surgical resection of pulmonary metastases from colorectal cancer should be assessed. Pfannschmidt *et al*[35] systematically reviewed 20 published series of surgical resection of pulmonary metastases from colorectal cancer. The postoperative mortality ranged from 0% to 2.4%, and approximately 40% of patients remained alive 5 years after resection. Fiorentino *et al*[36] also performed a systematic review of 51 articles on pulmonary metastasectomy in colorectal cancer. Most pulmonary metastasectomies were performed for a single metastasis. The 5-year survival rate after single metastasectomy was approximately 50%, whereas the rate after multiple metastasectomy was 30%. Recently, Gonzalez *et al*[37] performed a systematic review of 25 studies involving a total of 2925 patients and found that the median 5-year survival rate was 43.5%. At present, data for long-term survival after RFA are too sparse to compare with surgical data, although the short- to mid-term survival data are promising. In our opinion, given the high local progression rate for tumors > 3 cm after RFA, patients with such tumors should undergo surgery whenever operable. As a therapy for small tumors, RFA may be competitive with metastasectomy, which must be validated in future trials.

ADVANTAGES AND DISADVANTAGES OF LUNG RFA

RFA has various notable advantages. The procedure is simple and can be performed percutaneously using local anesthesia. The procedure is also safe and minimally invasive. Thus, RFA may enable long-term survival or even a cure for patients with pulmonary metastases who are considered nonsurgical candidates because of comorbidities and/or refusal to undergo surgery. Given that pulmonary metastases are usually of a multifocal nature and, consequently, pose a high risk of intrapulmonary *de novo* recurrence after therapy, the treatment for pulmonary metastases must be repeatable and should preserve as much of the parenchyma as possible to preserve pulmonary function. The repeatability of the procedure may also be a great advantage of RFA. Repeat procedures may also be used to effectively treat local tumor progression[38]. The influence of RFA on pulmonary function was found to be minimal[39-41], and RFA may be applied regardless of previous treatments. Consequently, this method can be used as a second salvage treatment for recurrence after surgery, radiation therapy, or chemotherapy and in combination with other treatments to eradicate multiple cancers.

There are also disadvantages of the use of RFA. CT is used for the procedure, which is associated with radiation exposure to both the patient and the physician. Thus, the use of CT fluoroscopy, although useful, should be minimized. The procedure is also accompanied by a high risk of pneumothorax. The most substantial disadvantage of RFA may be its limited local efficacy.

CONCLUSION

RFA for pulmonary metastasis of colorectal cancer is safe and minimally invasive. The most common complication, which occurs in up to 50% of cases, is pneumothorax. However, in most cases, this can be treated conservatively. The local efficacy of RFA depends on the tumor size, and local progression after RFA is not rare, occurring in 10% or more of cases. The local progression rate is particularly high for tumors > 3 cm. The short- to mid-term survival data after RFA are promising, with 1- and 3-year survival rates of approximately 85%-95% and 45%-55%, respectively. Long-term survival data remain sparse. Better survival may be expected for patients with small metastasis, low carcinoembryonic antigen levels, and/or no extrapulmonary metastasis. The ultimate application of RFA may be to replace metastasectomy for small metastases. Future studies should include randomized trials comparing RFA with surgery.

REFERENCES

1 **Jemal A**, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ. Cancer statistics, 2006. *CA Cancer J Clin* 2006; **56**: 106-130 [PMID: 16514137 DOI: 10.3322/canjclin.56.2.106]

2 **Mitry E**, Guiu B, Cosconea S, Jooste V, Faivre J, Bouvier AM. Epidemiology, management and prognosis of colorectal cancer with lung metastases: a 30-year population-based study. *Gut* 2010; **59**: 1383-1388 [PMID: 20732912 DOI: 10.1136/gut.2010.211557]

3 **Kobayashi H**, Mochizuki H, Sugihara K, Morita T, Kotake K, Teramoto T, Kameoka S, Saito Y, Takahashi K, Hase K, Oya M, Maeda K, Hirai T, Kameyama M, Shirouzu K, Muto T. Characteristics of recurrence and surveillance tools after curative resection for colorectal cancer: a multicenter study. *Surgery* 2007; **141**: 67-75 [PMID: 17188169 DOI: 10.1016/

j.surg.2006.07.020]
4 **McCormack PM**, Ginsberg RJ. Current management of colorectal metastases to lung. *Chest Surg Clin N Am* 1998; **8**: 119-126 [PMID: 9515176]
5 **Manfredi S**, Bouvier AM, Lepage C, Hatem C, Dancourt V, Faivre J. Incidence and patterns of recurrence after resection for cure of colonic cancer in a well defined population. *Br J Surg* 2006; **93**: 1115-1122 [PMID: 16804870 DOI: 10.1002/bjs.5349]
6 **Simmonds PC**. Palliative chemotherapy for advanced colorectal cancer: systematic review and meta-analysis. Colorectal Cancer Collaborative Group. *BMJ* 2000; **321**: 531-535 [PMID: 10968812 DOI: 10.1136/bmj.321.7260.531]
7 Long-term results of lung metastasectomy: prognostic analyses based on 5206 cases. The International Registry of Lung Metastases. *J Thorac Cardiovasc Surg* 1997; **113**: 37-49 [PMID: 9011700 DOI: 10.1016/S0022-5223(97)70397-0]
8 **Nahum Goldberg S**, Dupuy DE. Image-guided radiofrequency tumor ablation: challenges and opportunities--part I. *J Vasc Interv Radiol* 2001; **12**: 1021-1032 [PMID: 11535764 DOI: 10.1016/S1051-0443(07)61587-5]
9 **Dupuy DE**, Zagoria RJ, Akerley W, Mayo-Smith WW, Kavanagh PV, Safran H. Percutaneous radiofrequency ablation of malignancies in the lung. *AJR Am J Roentgenol* 2000; **174**: 57-59 [PMID: 10628454 DOI: 10.2214/ajr.174.1.1740057]
10 **Ahmed M**, Liu Z, Afzal KS, Weeks D, Lobo SM, Kruskal JB, Lenkinski RE, Goldberg SN. Radiofrequency ablation: effect of surrounding tissue composition on coagulation necrosis in a canine tumor model. *Radiology* 2004; **230**: 761-767 [PMID: 14990840 DOI: 10.1148/radiol.2303021801]
11 **Iishi T**, Hiraki T, Mimura H, Gobara H, Kurose T, Fujiwara H, Sakurai J, Yanai H, Yoshino T, Kanazawa S. Infusion of hypertonic saline into the lung parenchyma during radiofrequency ablation of the lungs with multitined expandable electrodes: results using a porcine model. *Acta Med Okayama* 2009; **63**: 137-144 [PMID: 19571900]
12 **Anai H**, Uchida BT, Pavcnik D, Seong CK, Baker P, Correa LO, Corless CL, Geyik S, Yavuz K, Sakaguchi H, Kichikawa K, Keller FS, Rösch J. Effects of blood flow and/or ventilation restriction on radiofrequency coagulation size in the lung: an experimental study in swine. *Cardiovasc Intervent Radiol* 2006; **29**: 838-845 [PMID: 16779691 DOI: 10.1007/s00270-005-0217-7]
13 **Iguchi T**, Hiraki T, Gobara H, Mimura H, Fujiwara H, Tajiri N, Sakurai J, Yasui K, Date H, Kanazawa S. Percutaneous radiofrequency ablation of lung tumors close to the heart or aorta: evaluation of safety and effectiveness. *J Vasc Interv Radiol* 2007; **18**: 733-740 [PMID: 17538135 DOI: 10.1016/j.jvir.2007.02.024]
14 **Rose SC**, Thistlethwaite PA, Sewell PE, Vance RB. Lung cancer and radiofrequency ablation. *J Vasc Interv Radiol* 2006; **17**: 927-51; quiz 951 [PMID: 16778226 DOI: 10.1097/01.RVI.0000222707.44902.66]
15 **Lencioni R**, Crocetti L, Cioni R, Suh R, Glenn D, Regge D, Helmberger T, Gillams AR, Frilling A, Ambrogi M, Bartolozzi C, Mussi A. Response to radiofrequency ablation of pulmonary tumours: a prospective, intention-to-treat, multicentre clinical trial (the RAPTURE study). *Lancet Oncol* 2008; **9**: 621-628 [PMID: 18565793 DOI: 10.1016/S1470-2045(08)70155-4]
16 **Hiraki T**, Gobara H, Mimura H, Toyooka S, Fujiwara H, Yasui K, Sano Y, Iguchi T, Sakurai J, Tajiri N, Mukai T, Matsui Y, Kanazawa S. Radiofrequency ablation of lung cancer at Okayama University Hospital: a review of 10 years of experience. *Acta Med Okayama* 2011; **65**: 287-297 [PMID: 22037265]
17 **Alafate A**, Shinya T, Okumura Y, Sato S, Hiraki T, Ishii H, Gobara H, Kato K, Fujiwara T, Miyoshi S, Kaji M, Kanazawa S. The Maximum standardized uptake value is more reliable than size measurement in early follow-up to evaluate potential pulmonary malignancies following radiofrequency ablation. *Acta Med Okayama* 2013; **67**: 105-112 [PMID: 23603927]
18 **Steinke K**, King J, Glenn D, Morris DL. Radiologic appearance and complications of percutaneous computed tomography-guided radiofrequency-ablated pulmonary metastases from colorectal carcinoma. *J Comput Assist Tomogr* 2003; **27**: 750-757 [PMID: 14501366 DOI: 10.1097/00004728-200309000-00012]
19 **Bojarski JD**, Dupuy DE, Mayo-Smith WW. CT imaging findings of pulmonary neoplasms after treatment with radiofrequency ablation: results in 32 tumors. *AJR Am J Roentgenol* 2005; **185**: 466-471 [PMID: 16037522 DOI: 10.2214/ajr.185.2.01850466]
20 **Abtin FG**, Eradat J, Gutierrez AJ, Lee C, Fishbein MC, Suh RD. Radiofrequency ablation of lung tumors: imaging features of the postablation zone. *Radiographics* 2012; **32**: 947-969 [PMID: 22786987 DOI: 10.1148/rg.324105181]
21 **Deandreis D**, Leboulleux S, Dromain C, Auperin A, Coulot J, Lumbroso J, Deschamps F, Rao P, Schlumberger M, de Baère T. Role of FDG PET/CT and chest CT in the follow-up of lung lesions treated with radiofrequency ablation. *Radiology* 2011; **258**: 270-276 [PMID: 21045185 DOI: 10.1148/radiol.10092440]
22 **Sharma A**, Lanuti M, He W, Palmer EL, Shepard JA, Digumarthy SR. Increase in fluorodeoxyglucose positron emission tomography activity following complete radiofrequency ablation of lung tumors. *J Comput Assist Tomogr* 2013; **37**: 9-14 [PMID: 23321827 DOI: 10.1097/RCT.0b013e3182732341]
23 **King J**, Glenn D, Clark W, Zhao J, Steinke K, Clingan P, Morris DL. Percutaneous radiofrequency ablation of pulmonary metastases in patients with colorectal cancer. *Br J Surg* 2004; **91**: 217-223 [PMID: 14760671]
24 **Steinke K**, Glenn D, King J, Clark W, Zhao J, Clingan P, Morris DL. Percutaneous imaging-guided radiofrequency ablation in patients with colorectal pulmonary metastases: 1-year follow-up. *Ann Surg Oncol* 2004; **11**: 207-212 [PMID: 14761926 DOI: 10.1245/ASO.2004.04.008]
25 **Yan TD**, King J, Sjarif A, Glenn D, Steinke K, Morris DL. Percutaneous radiofrequency ablation of pulmonary metastases from colorectal carcinoma: prognostic determinants for survival. *Ann Surg Oncol* 2006; **13**: 1529-1537 [PMID: 17009153 DOI: 10.1245/s10434-006-9101-1]
26 **Yan TD**, King J, Sjarif A, Glenn D, Steinke K, Al-Kindy A, Morris DL. Treatment failure after percutaneous radiofrequency ablation for nonsurgical candidates with pulmonary metastases from colorectal carcinoma. *Ann Surg Oncol* 2007; **14**: 1718-1726 [PMID: 17285398 DOI: 10.1245/s10434-006-9271-x]
27 **Yan TD**, King J, Sjarif A, Glenn D, Steinke K, Morris DL. Learning curve for percutaneous radiofrequency ablation of pulmonary metastases from colorectal carcinoma: a prospective study of 70 consecutive cases. *Ann Surg Oncol* 2006; **13**: 1588-1595 [PMID: 16957970 DOI: 10.1245/s10434-006-9010-3]
28 **Chua TC**, Sarkar A, Saxena A, Glenn D, Zhao J, Morris DL. Long-term outcome of image-guided percutaneous radiofrequency ablation of lung metastases: an open-labeled prospective trial of 148 patients. *Ann Oncol* 2010; **21**: 2017-2022 [PMID: 20335366 DOI: 10.1093/annonc/mdq098]
29 **Simon CJ**, Dupuy DE, DiPetrillo TA, Safran HP, Grieco CA, Ng T, Mayo-Smith WW. Pulmonary radiofrequency ablation: long-term safety and efficacy in 153 patients. *Radiology* 2007; **243**: 268-275 [PMID: 17392258 DOI: 10.1148/radiol.2431060088]
30 **Hiraki T**, Gobara H, Iishi T, Sano Y, Iguchi T, Fujiwara H, Tajiri N, Sakurai J, Date H, Mimura H, Kanazawa S. Percutaneous radiofrequency ablation for pulmonary metastases from colorectal cancer: midterm results in 27 patients. *J Vasc Interv Radiol* 2007; **18**: 1264-1269 [PMID: 17911517 DOI: 10.1016/j.jvir.2007.06.027]
31 **Yamakado K**, Hase S, Matsuoka T, Tanigawa N, Nakatsuka A, Takaki H, Takao M, Inoue Y, Kanazawa S, Inoue

Y, Sawada S, Kusunoki M, Takeda K. Radiofrequency ablation for the treatment of unresectable lung metastases in patients with colorectal cancer: a multicenter study in Japan. *J Vasc Interv Radiol* 2007; **18**: 393-398 [PMID: 17377185 DOI: 10.1016/j.jvir.2006.11.003]

32 **Yamakado K**, Inoue Y, Takao M, Takaki H, Nakatsuka A, Uraki J, Kashima M, Kusunoki M, Shimpo H, Takeda K. Long-term results of radiofrequency ablation in colorectal lung metastases: single center experience. *Oncol Rep* 2009; **22**: 885-891 [PMID: 19724869 DOI: 10.3892/or_00000513]

33 **Petre EN**, Jia X, Thornton RH, Sofocleous CT, Alago W, Kemeny NE, Solomon SB. Treatment of pulmonary colorectal metastases by radiofrequency ablation. *Clin Colorectal Cancer* 2013; **12**: 37-44 [PMID: 23026111 DOI: 10.1016/j.clcc.2012.07.003]

34 **Gillams A**, Khan Z, Osborn P, Lees W. Survival after radiofrequency ablation in 122 patients with inoperable colorectal lung metastases. *Cardiovasc Intervent Radiol* 2013; **36**: 724-730 [PMID: 23070108 DOI: 10.1007/s00270-012-0500-3]

35 **Pfannschmidt J**, Dienemann H, Hoffmann H. Surgical resection of pulmonary metastases from colorectal cancer: a systematic review of published series. *Ann Thorac Surg* 2007; **84**: 324-338 [PMID: 17588454 DOI: 10.1016/j.athoracsur.2007.02.093]

36 **Fiorentino F**, Hunt I, Teoh K, Treasure T, Utley M. Pulmonary metastasectomy in colorectal cancer: a systematic review and quantitative synthesis. *J R Soc Med* 2010; **103**: 60-66 [PMID: 20118336 DOI: 10.1258/jrsm.2009.090299]

37 **Gonzalez M**, Poncet A, Combescure C, Robert J, Ris HB, Gervaz P. Risk factors for survival after lung metastasectomy in colorectal cancer patients: a systematic review and meta-analysis. *Ann Surg Oncol* 2013; **20**: 572-579 [PMID: 23104709 DOI: 10.1245/s10434-012-2726-3]

38 **Hiraki T**, Mimura H, Gobara H, Sano Y, Fujiwara H, Date H, Kanazawa S. Repeat radiofrequency ablation for local progression of lung tumors: does it have a role in local tumor control? *J Vasc Interv Radiol* 2008; **19**: 706-711 [PMID: 18440459 DOI: 10.1016/j.jvir.2007.12.441]

39 **Ambrogi MC**, Lucchi M, Dini P, Melfi F, Fontanini G, Faviana P, Fanucchi O, Mussi A. Percutaneous radiofrequency ablation of lung tumours: results in the mid-term. *Eur J Cardiothorac Surg* 2006; **30**: 177-183 [PMID: 16723242 DOI: 10.1016/j.ejcts.2006.03.067]

40 **Tada A**, Hiraki T, Iguchi T, Gobara H, Mimura H, Toyooka S, Kiura K, Tsuda T, Mitsuhashi T, Kanazawa S. Influence of radiofrequency ablation of lung cancer on pulmonary function. *Cardiovasc Intervent Radiol* 2012; **35**: 860-867 [PMID: 21725672 DOI: 10.1007/s00270-011-0221-z]

41 **de Baère T**, Palussière J, Aupérin A, Hakime A, Abdel-Rehim M, Kind M, Dromain C, Ravaud A, Tebboune N, Boige V, Malka D, Lafont C, Ducreux M. Midterm local efficacy and survival after radiofrequency ablation of lung tumors with minimum follow-up of 1 year: prospective evaluation. *Radiology* 2006; **240**: 587-596 [PMID: 16864679 DOI: 10.1148/radiol.2402050807]

P- Reviewers: Bloomston PM, Borzio M, Ewertsen C
S- Editor: Zhai HH **L- Editor**: A **E- Editor**: Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.997

World J Gastroenterol 2014 January 28; 20(4): 997-1004
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (5): Colorectal cancer

Overview of single-port laparoscopic surgery for colorectal cancers: Past, present, and the future

Say-June Kim, Byung-Jo Choi, Sang Chul Lee

Say-June Kim, Byung-Jo Choi, Sang Chul Lee, Department of Surgery, Daejeon St. Mary's Hospital, the Catholic University of Korea, Daeheung-dong 520-2, Jung-gu, Daejeon 301-723, South Korea
Author contributions: Lee SC designed the study, and finally approved the version to be published; Choi BJ contributed to acquiring and analyzing data; Kim SJ wrote the paper.
Correspondence to: Sang-Chul Lee, MD, PhD, Department of Surgery, Daejeon St. Mary's Hospital, the Catholic University of Korea, Daeheung-dong 520-2, Jung-gu, Daejeon 301-723, South Korea. zambo9@catholic.ac.kr
Telephone: +82-42-2209269 Fax: +82-42-2209565
Received: August 31, 2013 Revised: November 1, 2013
Accepted: December 5, 2013
Published online: January 28, 2014

Abstract

Single-port laparoscopic surgery (SPLS) is implemented through a tailored minimal single incision through which a number of laparoscopic instruments access. Introduction of operation-customized port system, utilization of a camera without a separate external light, and instruments with different lengths has brought the favorable environment for SPLS. However, performing SPLS still creates several hardships compared to multiport laparoscopic surgery; a single-port system inevitably leads to clashing of surgical instruments due to crowding. To overcome such difficulties, investigators has developed novel concepts and maneuvers, including the concept of inverse triangulation and the maneuvers of pivoting, spreading out dissection, hanging suture, and transluminal traction. The final destination of SPLS is expected to be a completely seamless operation, maximizing the minimal invasiveness. Specimen extraction through the umbilicus can undermine cosmesis by inducing a larger incision. Therefore, hybrid laparoscopic technique, which combined laparoscopic surgical technique with natural orifice specimen extraction (NOSE) - *i.e.*, transvaginal or transanal route-, has been developed. SPLS and NOSE seemed to be the best combination in pursuit of minimal invasiveness. In the near future, robotic SPLS with natural orifice transluminal endoscopic surgery's way of specimen extraction seems to be pursued. It is expected to provide a completely or nearly complete seamless operation regardless of location of the lesion in the abdomen.

Key words: Colorectal neoplasms; Colectomy; Laparoscopy; Natural orifice endoscopic surgery; Single-port laparoscopic surgery

Core tip: Single-port laparoscopic surgery (SPLS) has clear-cut benefits in terms of cosmesis and reduced wound morbidity. The technical difficulties have been overcome by novel concepts and maneuvers, including the concept of inverse triangulation and the maneuvers of pivoting, spreading out dissection, hanging suture, and transluminal traction. Cosmetic demerits, caused by the specimen extraction through the single-port site, can be selectively overcome by natural orifice specimen extraction, such as using transvaginal or transanal route. In the near future, robotic SPLS with natural orifice transluminal endoscopic surgery's way of specimen extraction seems to be pursued.

Kim SJ, Choi BJ, Lee SC. Overview of single-port laparoscopic surgery for colorectal cancers: Past, present, and the future. *World J Gastroenterol* 2014; 20(4): 997-1004 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/997.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.997

PAST: EMERGING AS A RISING HOPE

Laparoscopic surgery did not only cosmetically satisfy patients but also led to improvement in parameters re-

Table 1 Acronyms of single port laparoscopic surgery

Acronym	Details
eNOTES	Embryonic natural orifice transluminal endoscopic surgery
LESS	Laparo-endoscopic single site surgery
NOTUS	Natural orifice trans-umbilical surgery
OPUS	One port umbilical surgery
SPAS	Single port access
SPL	Single port laparoscopy
SIPLS	Single instrument port laparoscopic surgery
SIMPL	Single incision multi-port laparoscopic-endoscopic
SILS	Single incision laparoscopic surgery
SLIT	Single laparoscopic incision transabdominal
SLAPP	Single laparoscopic port procedure
SSL	Single site laparoscopy
TUES	Trans-umbilical endoscopic surgery
TULA	Trans-umbilical laparoscopic assisted
TUSPLS	trans-umbilical single port laparoscopic surgery

lated with short-term operative outcomes, such as reduction in postoperative pain and duration of ileus, quicker postoperative recovery, shorter hospital stay, and earlier return to normal activity[1-7]. Furthermore, a randomized clinical trial reported a reduction in tumor relapse following laparoscopic surgery, suggesting long-term oncologic benefits[8]. The reasons were attributed to various potential mechanisms, including a lower stress response after surgical trauma, an attenuated cytokine response, minimal tumor handling, accurate application of the no-touch technique, and lower complication rates.

Conventional laparoscopic surgery (CLS) usually requires 3-6 small incisions for ports. These incisions are not only cosmetically unappealing, but also increase the wound pain and potential wound morbidity, such as abdominal wall bleeding, port-site hernia, and internal organ damage. The ardent pursuit of minimal invasiveness and the increasing recognition of patient's satisfaction has led to the ultimate form of laparoscopic surgery, single port laparoscopic surgery (SPLS). Ever since the first attempt of SPLS hysterectomy in 1992[9], SPLS was adopted by general surgery in procedures such as appendectomy[10], cholecystectomy[11], and adrenalectomy[12]. Colorectal surgeons were also eager to employ the novel SPLS technique in right hemicolectomy[13,14], sigmoidectomy[15,16], and total colectomy[17,18]. The spectrum of SPLS applications has extended from benign diseases to malignant colorectal cancers[13,16,19] and the safety and feasibility of SPLS in colorectal surgery is supported by many reports and comparative studies[14,20-22].

PRESENT: EXCLAMATION AND FRUSTRATION

SPLS nomenclature

The exact nomenclature of laparoscopic surgery, which is performed through only on minimal incision, has not been determined. The surgical procedure has been variously referred depending on the continent, country, hospital, department, and even individual operator (Table 1). In this paper, we referred to it as SPLS, which is the most widely used terminology in South Korea.

Beneficial effects of SPLS

SPLS is implemented through a tailored minimal single incision through which a number of laparoscopic instruments access. This single incision site usually functions as (1) an access port entering into abdominal cavity; (2) a specimen-extracting orifice; and (3) a pathway for a drain. The preferred single incision site is the umbilicus. Umbilicus is the thinnest part of the abdomen; has no vessel or nerve; and can be regarded as predetermined, ready-made scar which can hide artificial scar effectively. Furthermore, centrally located, it can provide a shortcut to various intra-abdominal organs in all abdominal quadrants. Other sites besides umbilicus, however, can be utilized as a single incision for various reasons, including abandoning the umbilicus due to possible adhesion and making incision at predetermined ileostomy site. We experienced several cases of abdominoperineal resection and low anterior resection using SPLS other than transumbilical route due to the latter reason, and found it to be acceptable in terms of operative proficiency and cosmesis[15]; no wound was identified postoperatively except for the ileostomy site, simulating an even more "scar-less" operation than using the umbilicus.

Besides cosmetic superiority, the potential benefits of SPLS is to reduce wound morbidity. The number and overall size of the wound directly affect wound morbidity, such as injuries of vessels, bowel, and other intra-abdominal organs, and trocar site hernia[23]. Weiss *et al*[24], in their analysis of 1145 consecutive series of SPLS, reported that SPLS reduced wound complication more than CLS (2.38% *vs* 8.45%, $P = 0.015$).

Other benefits of SPLS over CLS have not determined yet. Until now, a series of comparative studies suggested a number of potential benefits of SPLS, including pain reduction and fastened postoperative recovery[20,25-27], and others did not[28-31]. The severity and duration of pain after an operation influences postoperative recovery, which is reflected by duration before re-initiation of a diet, return to normal activity, and the length of hospital stay. Therefore, the effect of SPLS on postoperative pain needs to be determined first. Tsimoyiannis *et al*[25], in a randomized controlled trial comparing outcomes following cholecystectomies either by CLS ($n = 20$) or SPLS ($n = 20$), showed that SPLS more reduced postoperative pain scores. However, prospective, large-scaled clinical trials of the short- and long-term outcomes are essential to determine the precise effects of SPLS.

SPLS is particularly useful in operations which are aimed at more than two target organs in different quadrants; for the umbilicus provides a shortcut to reach all intra-abdominal organs. Combined appendectomy and cholecystectomy is one of examples. Colorectal surgery involves the most extensive area in the abdomen because the colorectum is extensively distributed. Therefore, the

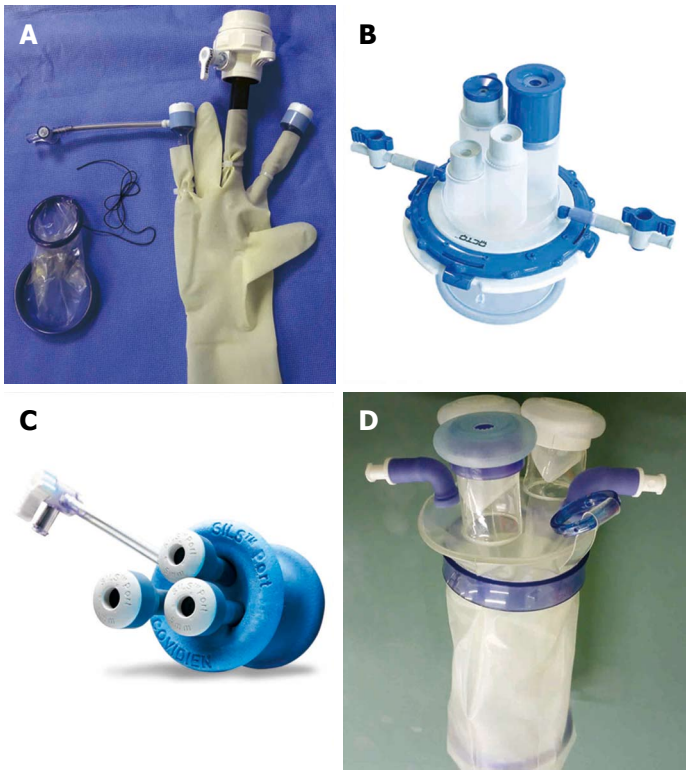


Figure 1 Ports designed for single-port laparoscopic surgery. A: Materials for making homemade glove port (two-piece, terminal type); B: OCTO port (Dalim medical Co., South Korea) (two-piece, terminal type); C: Single incision laparoscopic surgery port (Covidien, United States) (one-piece, preoccupied type); D: Commercial glove port (Sejong medical Co., South Korea) (one-piece, terminal type).

merit of SPLS is pronounced in colorectal surgery. Furthermore, SPLS may be the optimal choice in selected patients with a history of multiple abdominal operations. Open or laparoscopic surgery can equally put such patients in the risk of iatrogenic bowel perforation. In these situations, SPLS can be attempted because a single minimal incision provides a safe settlement point from which the dissection can be initiated cautiously.

SPLS poses several challenges, such as the handling of straight instruments in parallel with the laparoscope through a small single incision. Technical limitations of instrumentation in SPLS has led to advancement in techniques to overcome the limitations. Such technical advancements are unique to SPLS; difficult or unable to apply to CLS; and therefore show the potential of SPLS to outperform CLS.

Instrument for SPLS

Ports: In the beginning, a homemade glove port, which combines a wound retractor and a surgical glove, has been utilized. More recently, commercial single ports, including the OCTO port (Dalim medical Co., South Korea) and the SILS port (single-incision laparoscopic surgery port, Covidien, United States) have also been developed and introduced (Figure 1). For convenience, we categorized the single ports into two subtypes depending on detachability; one-piece (SPLS port, R-port *etc.*) type and two-piece (Glove port, OCTO port *etc.*) type. We think that the two-piece type is more convenient in colorectal surgery considering the comfortability of specimen extraction through the port site. We also classified the single ports into the terminal type and preoccupied type according to the presence of a common channel; it can be called as terminal type when laparoscopic instruments share a common channel in the single port except for their entrance, and called as preoccupied type when each individual laparoscopic instrument has its own independent access to the abdominal cavity. We preferred the terminal type because it can be used with smaller incisions and evokes less instrumental clinching.

Camera: Utilization of a camera without a separate external light not only provides more space externally but also reduces the chance of it being knocked out of place by a surgeon. We prefer a camera with 5-mm diameter due to various reasons, such as taking up lesser space and leaving small incision. The 30-degree telescope provides an extensive vision, especially in the deepest portion of the pelvic cavity.

Working instruments: As SPLS is based on well-established laparoscopic foundation, SPLS can be reproduced using conventional laparoscopic instruments. Fixed straight instruments are usually preferred in SPLS because they can transmit constant force and maintain throughout retraction. Numerous articulating devices, however, have been developed to actively manipulate and fulfill tasks regardless of instrument position. The prac-

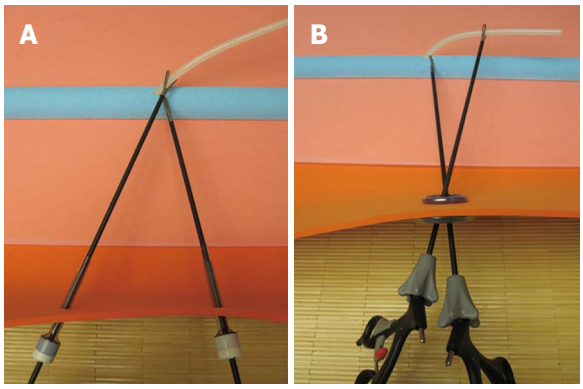


Figure 2 Concept of triangulation *vs* inverse triangulation. Triangulation in multiport laparoscopic surgery allows traction on tissues to improve dissection along anatomical planes (A). In inverse triangulation of single-port laparoscopic surgery, the two instrumental ends do not encounter, but assist each other by creating tension (B). Therefore, the operation is carried out with the two instruments crossed-over.

tical utility of their flexibility raises controversy. Judging from our experience, articulating devices were particularly convenient when utilized with one hand rather than with both hands and/or when applied to the patients with a prominent pelvic promontory. Instruments with longer (44-45 cm) shaft lengths than conventional devices (33-34 cm) are advantageous in the procedures in the left upper quadrant (LUQ), such as splenic flexure dissection. Considerable instrumental clinches occurred outside rather than inside of abdominal cavity. We have overcome the clinches to a large extent using different-length instruments and a reduced bulk camera.

An instrument placed through a single port divides the hole in the port into two. Therefore, when an instrument cannot, or is difficult to reach the targeted organ, we recommend to draw the instrument completely out of the abdomen and re-insert it into another compartment bordered by the instrument.

Challenge and response

Performing SPLS is more strenuous than CLS. The environment provided by SPLS inevitably results in motion limitations and clashing of surgical instruments due to crowding. Furthermore, SPLS significantly increases the difficulty of colonic exposure and dissection due to inability of triangular dissection which has been considered a cornerstone of laparoscopic surgery. Such difficulties prompted the development of instruments and maneuvers to overcome the limitations. Herein, some of these attempts, including the maneuvers which were ingenuously developed at our institution, will be discussed.

Inverse triangulation: Laparoscopic surgeons have performed convenient traction and dissection using the concept of triangulation. SPLS, however, provides the unfavorable surroundings for triangulation, often resulting in the chopsticks or sword fighting effect due to parallel alignment of instruments. We have attempted to overcome the limitations using a new concept of "inverse triangulation" (Figure 2). Inverse triangulation refers to the formation of an inverted triangle viewed from the operator; one single-incision port site and two instrumental ends which are positioned in a crossing-over pattern comprise three triangles. The two instrumental ends do not encounter, but assist each other by creating tension. The operation is carried out with the two instruments crossed-over. The surgeon's right hand holds the left-sided instrument and vice versa. Inverse-triangulation makes it convenient to perform various kinds of laparoscopic procedure, including dissection, traction, and resection. And, inverse triangulation does not increase the umbilical pain because the range of motion of the instruments is restricted within the umbilical port.

Pivoting: Colorectum is located extensively in four quadrants of the abdomen, and the umbilicus is located in the center of four quadrants. Therefore, a pan-abdominal approach without additional incisions is possible through the umbilicus. Furthermore, SPLS is advantageous in the operation which includes more than two target organs in different quadrants, such as combining splenectomy and appendectomy. The only requirements in such a situation are positional changes of the patient and shifts of operation members.

Spreading out dissection: Whether it is laparoscopy or open surgery, the operation of the patient with multiple adhesions demands a great deal of hard works. In CLS, even if a port for camera is successfully entered, insertion of an additional port far apart from the camera port can be threatening due to potential risk of intestinal injuries. However, SPLS has an advantage over CLS in that it does not require risky additional port insertion; only after securement of single-port access, dissection of adherent tissue can be expanded from the single-port site with safety.

Hanging suture: Application of a hanging suture is helpful when sustained maintenance of the visual field overcoming an obstacle is required. For example, the practice of total mesorectal excision (TME) for rectal cancer is limited during SPLS due to narrow pelvic cavity and hindering structures. To facilitate TME, the peritoneal fold (in males) or the uterus (in females) can be elevated by placing an intracorporeal stitch through the low abdominal wall (Figure 3). Thereafter, adjusting patient's position according to the procedure can further optimize operative field.

Transluminal traction: In the low colorectal surgery, the support of a colorectum, which is determined to be dissected, can facilitate dissection by way of adjusting the organ's direction. This support can be provided by transrectal application of instruments, such as PPH (Procedure for Prolapsed and Hemorrhoid Endo-Surgery, Ethicon, United States), a circular stapler, an anal trocar, or colonoscopy (Figure 4).

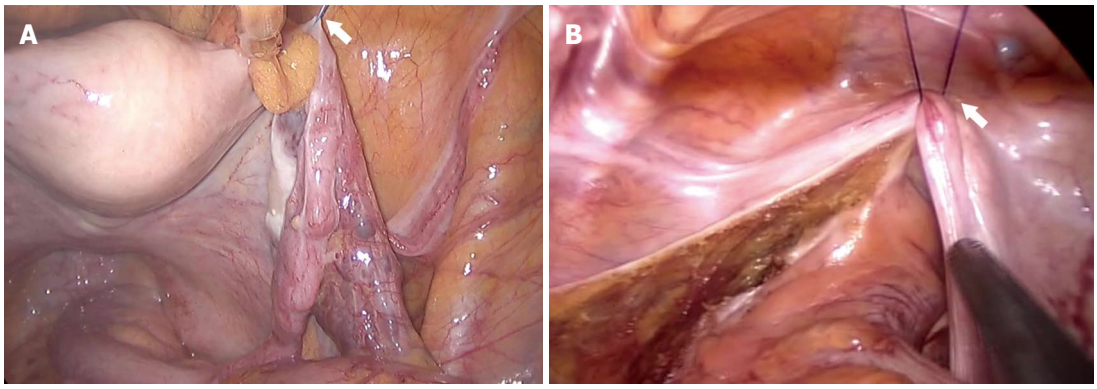


Figure 3 Hanging suture. To facilitate operative field during total mesorectal excision, the uterus in female (A) or the peritoneal fold in male (B) were elevated by placing an intracorporeal stitch through the low abdominal wall.

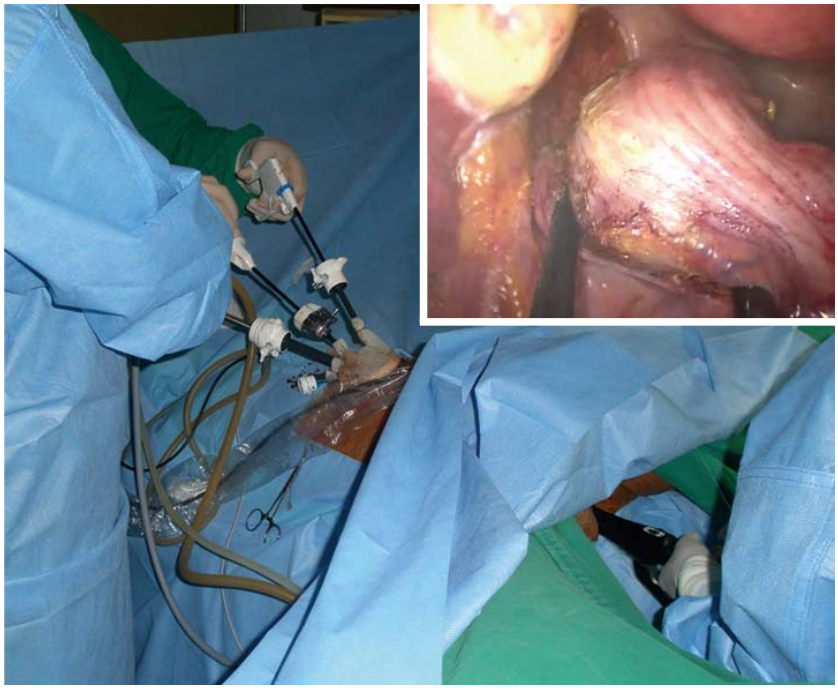


Figure 4 Transluminal traction. A PPH (procedure for Prolapsed and Hemorrhoid Endo-Surgery, Ethicon, United States) was utilized to support the colorectum during dissection and to facilitate dissection by shifting the colorectum's location as well.

FUTURE: WAY TO ULTIMITE SCARLESS SURGERY

SPLS is mainly accomplished by two persons. The operator both holds an organ structure and dissects it with bimanual manipulation. Therefore, the operator's contribution is more substantial than any other procedures. An assistant's role is, however, usually to steer a laparoscope. Therefore, the assistant's role can be replaced by an instrument, such as a camera holder (laparoscopic instrument holder, Sejong medical Co., South Korea) (Figure 5). If the instrument replaces an assistant surgeon, the surgical team is only comprised of a surgeon and a scrub nurse. Surgery department often lacks manpower; therefore such instrument-dependent SPLS can overcome the personal defect. We found it is particularly advantageous in the operations of which target organ is localized in a single quadrant, such as appendectomy, cholecystectomy, and herniorrhaphy.

SPLS was initially designed to achieve a seamless operation. Reaching a "completely seamless operation" is the final destination, while maintaining comparable therapeutic outcomes as CLS. In spite of attempts to reduce the number and size of the skin incision, the bulk of the specimen inevitably affects the length of incision, mostly the umbilical incision. Laparoscopic surgeons attempted to solve this problem by borrowing idea from natural orifice transluminal endoscopic surgery (NOTES). Consequently, hybrid laparoscopic technique, which combined laparoscopic surgical technique with natural orifice specimen extraction (NOSE), has been developed[32-41]. Of NOSE, transvaginal[32-37] or transanal[38-41] route of

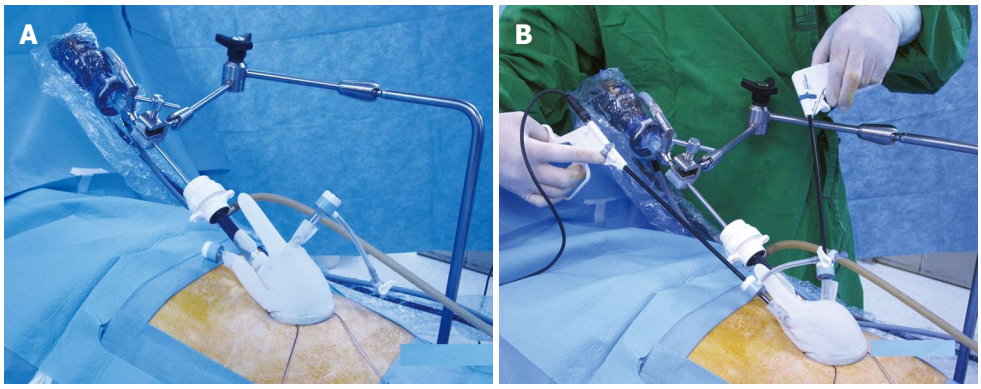


Figure 5 Laparoscopic instrument holder. An installation of a laparoscopic instrument holder in operation bed (A). Application of a laparoscopic instrument holder during single-port laparoscopic surgery (B).

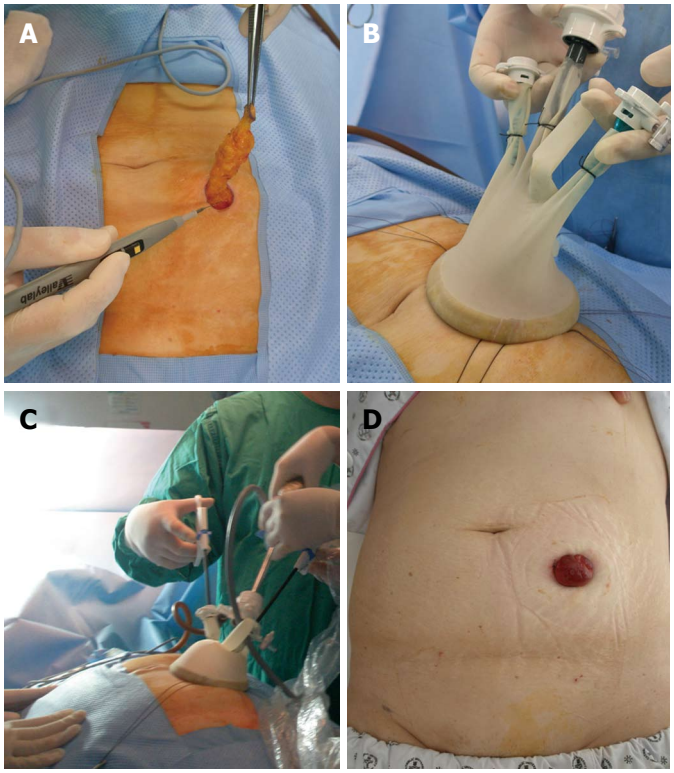


Figure 6 Umbilicus-sparing single-port laparoscopic surgery. After making a incision for single-port to the predetermined enterostomy site (A), a single-port was inserted (B), and operation was accomplished through the enterostomy site (C). Postoperatively, no scar, except for enterostomy, remained (D).

specimen retrieval has been preferred in laparoscopic surgery. SPLS and NOSE are the best combination in pursuit of minimal invasiveness. In operations which include the formation of a stoma, such as abdominoperineal resection or low anterior resection with diverting ileostomy, SPLS can be initiated through a predetermined stoma site and the specimen can be extracted through the stoma site (Figure 6). Therefore, the ideal seamless operation can be accomplished by this way.

Transanal endoluminal laparoscopic surgery (TELS) is displayed in the rectal lumen using a port established in the anus[42-44]. And, laparoscopic assisted transanal transabdominal proctosigmoidectomy is a combined approach to remove low rectal cancer *via* the anus and abdominal cavity[45-48]. Ideal seamless operation can be designed by combining these two operative procedures. First, after making an incision in anus, the dissection proceeds forward, and then the specimen is extracted *via* anus, and colo-anal anastomosis is achieved through the anus. Such an accomplishment can be remarked as one of the most advanced forms of SPLS[49,50].

The advent of robotic surgery should be addressed when discussing the future of minimally invasive surgery. Because the robotic surgery is performed using a laparoscopic approach, an upgraded version of robotic surgery will be single-port robotic surgery[51]. It seemed that robotic SPLS combined with NOTE's way of specimen extraction would be attempted in the near future[52].

It is expected to provide a completely or nearly complete seamless operation regardless of location of the lesion in the abdomen.

REFERENCES

1 **Braga M**, Vignali A, Gianotti L, Zuliani W, Radaelli G, Gruarin P, Dellabona P, Di Carlo V. Laparoscopic versus open colorectal surgery: a randomized trial on short-term outcome. *Ann Surg* 2002; **236**: 759-766; disscussion 767 [PMID: 12454514 DOI: 10.1097/01.SLA.0000036269.60340.AE]

2 **Clinical Outcomes of Surgical Therapy Study Group**. A comparison of laparoscopically assisted and open colectomy for colon cancer. *N Engl J Med* 2004; **350**: 2050-2059 [PMID: 15141043 DOI: 10.1056/NEJMoa032651]

3 **Fleshman J**, Sargent DJ, Green E, Anvari M, Stryker SJ, Beart RW, Hellinger M, Flanagan R, Peters W, Nelson H. Laparoscopic colectomy for cancer is not inferior to open surgery based on 5-year data from the COST Study Group trial. *Ann Surg* 2007; **246**: 655-662; discussion 662-664 [PMID: 17893502 DOI: 10.1097/SLA.0b013e318155a762]

4 **Guillou PJ**, Quirke P, Thorpe H, Walker J, Jayne DG, Smith AM, Heath RM, Brown JM. Short-term endpoints of conventional versus laparoscopic-assisted surgery in patients with colorectal cancer (MRC CLASICC trial): multicentre, randomised controlled trial. *Lancet* 2005; **365**: 1718-1726 [PMID: 15894098 DOI: 10.1016/S0140-6736(05)66545-2]

5 **Hewett PJ**, Allardyce RA, Bagshaw PF, Frampton CM, Frizelle FA, Rieger NA, Smith JS, Solomon MJ, Stephens JH, Stevenson AR. Short-term outcomes of the Australasian randomized clinical study comparing laparoscopic and conventional open surgical treatments for colon cancer: the ALCCaS trial. *Ann Surg* 2008; **248**: 728-738 [PMID: 18948799 DOI: 10.1097/SLA.0b013e31818b7595]

6 **Ng KH**, Ng DC, Cheung HY, Wong JC, Yau KK, Chung CC, Li MK. Laparoscopic resection for rectal cancers: lessons learned from 579 cases. *Ann Surg* 2009; **249**: 82-86 [PMID: 19106680 DOI: 10.1097/SLA.0b013e31818e418a]

7 **Veldkamp R**, Kuhry E, Hop WC, Jeekel J, Kazemier G, Bonjer HJ, Haglind E, Påhlman L, Cuesta MA, Msika S, Morino M, Lacy AM. Laparoscopic surgery versus open surgery for colon cancer: short-term outcomes of a randomised trial. *Lancet Oncol* 2005; **6**: 477-484 [PMID: 15992696 DOI: 10.1016/S1470-2045(05)70221-7]

8 **Lacy AM**, Delgado S, Castells A, Prins HA, Arroyo V, Ibarzabal A, Pique JM. The long-term results of a randomized clinical trial of laparoscopy-assisted versus open surgery for colon cancer. *Ann Surg* 2008; **248**: 1-7 [PMID: 18580199 DOI: 10.1097/SLA.0b013e31816a9d65]

9 **Pelosi MA**, Pelosi MA. Laparoscopic supracervical hysterectomy using a single-umbilical puncture (mini-laparoscopy). *J Reprod Med* 1992; **37**: 777-784 [PMID: 1453397]

10 **Ateş O**, Hakgüder G, Olguner M, Akgür FM. Single-port laparoscopic appendectomy conducted intracorporeally with the aid of a transabdominal sling suture. *J Pediatr Surg* 2007; **42**: 1071-1074 [PMID: 17560223 DOI: 10.1016/j.jpedsurg.2007.01.065]

11 **Podolsky ER**, Rottman SJ, Poblete H, King SA, Curcillo PG. Single port access (SPA) cholecystectomy: a completely transumbilical approach. *J Laparoendosc Adv Surg Tech A* 2009; **19**: 219-222 [PMID: 19260790 DOI: 10.1089/lap.2008.0275]

12 **Hirano D**, Minei S, Yamaguchi K, Yoshikawa T, Hachiya T, Yoshida T, Ishida H, Takimoto Y, Saitoh T, Kiyotaki S, Okada K. Retroperitoneoscopic adrenalectomy for adrenal tumors via a single large port. *J Endourol* 2005; **19**: 788-792 [PMID: 16190829 DOI: 10.1089/end.2005.19.788]

13 **Bucher P**, Pugin F, Morel P. Single port access laparoscopic right hemicolectomy. *Int J Colorectal Dis* 2008; **23**: 1013-1016 [PMID: 18607608 DOI: 10.1007/s00384-008-0519-8]

14 **Remzi FH**, Kirat HT, Kaouk JH, Geisler DP. Single-port laparoscopy in colorectal surgery. *Colorectal Dis* 2008; **10**: 823-826 [PMID: 18684153 DOI: 10.1111/j.1463-1318.2008.01660.x]

15 **Brunner W**, Schirnhofer J, Waldstein-Wartenberg N, Frass R, Weiss H. Single incision laparoscopic sigmoid colon resections without visible scar: a novel technique. *Colorectal Dis* 2010; **12**: 66-70 [PMID: 19508527 DOI: 10.1111/j.1463-1318.2009.01894.x]

16 **Bucher P**, Pugin F, Morel P. Transumbilical single incision laparoscopic sigmoidectomy for benign disease. *Colorectal Dis* 2010; **12**: 61-65 [PMID: 19320667 DOI: 10.1111/j.1463-1318.2009.01825.x]

17 **Leblanc F**, Makhija R, Champagne BJ, Delaney CP. Single incision laparoscopic total colectomy and proctocolectomy for benign disease: initial experience. *Colorectal Dis* 2011; **13**: 1290-1293 [PMID: 20955513 DOI: 10.1111/j.1463-1318.2010.02448.x]

18 **Cahill RA**, Lindsey I, Jones O, Guy R, Mortensen N, Cunningham C. Single-port laparoscopic total colectomy for medically uncontrolled colitis. *Dis Colon Rectum* 2010; **53**: 1143-1147 [PMID: 20628277 DOI: 10.1007/DCR.0b013e3181dd062f]

19 **Mufty H**, Hillewaere S, Appeltans B, Houben B. Single-incision right hemicolectomy for malignancy: a feasible technique with standard laparoscopic instrumentation. *Colorectal Dis* 2012; **14**: e764-e770 [PMID: 22776288 DOI: 10.1111/j.1463-1318.2012.03175.x]

20 **Kim SJ**, Ryu GO, Choi BJ, Kim JG, Lee KJ, Lee SC, Oh ST. The short-term outcomes of conventional and single-port laparoscopic surgery for colorectal cancer. *Ann Surg* 2011; **254**: 933-940 [PMID: 22107740 DOI: 10.1097/SLA.0b013e318237826b]

21 **Napolitano L**, Waku M, De Nicola P, Di Bartolomeo N, Cotellese R, D'Aulerio A, Innocenti P. Laparoscopic colectomy in colon cancer. A single-center clinical experience. *G Chir* 2007; **28**: 126-133 [PMID: 17475112]

22 **Singh J**, Podolsky ER, Castellanos AE, Stein DE. Optimizing single port surgery: a case report and review of technique in colon resection. *Int J Med Robot* 2011; **7**: 127-130 [PMID: 21394870 DOI: 10.1002/rcs.378]

23 **Shabanzadeh DM**, Sørensen LT. Laparoscopic surgery compared with open surgery decreases surgical site infection in obese patients: a systematic review and meta-analysis. *Ann Surg* 2012; **256**: 934-945 [PMID: 23108128 DOI: 10.1097/SLA.0b013e318269a46b]

24 **Weiss HG**, Brunner W, Biebl MO, Schirnhofer J, Pimpl K, Mittermair C, Obrist C, Brunner E, Hell T. Wound complications in 1145 consecutive transumbilical single-incision laparoscopic procedures. *Ann Surg* 2014; **259**: 89-95 [PMID: 23426333 DOI: 10.1097/SLA.0b013e31827b7818]

25 **Tsimoyiannis EC**, Tsimogiannis KE, Pappas-Gogos G, Farantos C, Benetatos N, Mavridou P, Manataki A. Different pain scores in single transumbilical incision laparoscopic cholecystectomy versus classic laparoscopic cholecystectomy: a randomized controlled trial. *Surg Endosc* 2010; **24**: 1842-1848 [PMID: 20174950 DOI: 10.1007/s00464-010-0887-3]

26 **Tugcu V**, Ilbey YO, Mutlu B, Tasci AI. Laparoendoscopic single-site surgery versus standard laparoscopic simple nephrectomy: a prospective randomized study. *J Endourol* 2010; **24**: 1315-1320 [PMID: 20626273 DOI: 10.1089/end.2010.0048]

27 **Yim GW**, Jung YW, Paek J, Lee SH, Kwon HY, Nam EJ, Kim S, Kim JH, Kim YT, Kim SW. Transumbilical single-port access versus conventional total laparoscopic hysterectomy: surgical outcomes. *Am J Obstet Gynecol* 2010; **203**: 26.e1-26.e6 [PMID: 20417481 DOI: 10.1016/j.ajog.2010.02.026]

28 **Ma J**, Cassera MA, Spaun GO, Hammill CW, Hansen PD, Aliabadi-Wahle S. Randomized controlled trial comparing single-port laparoscopic cholecystectomy and four-port laparoscopic cholecystectomy. *Ann Surg* 2011; **254**: 22-27 [PMID: 21494123 DOI: 10.1097/SLA.0b013e3182192f89]

29 **Lai EC**, Yang GP, Tang CN, Yih PC, Chan OC, Li MK. Prospective randomized comparative study of single incision laparoscopic cholecystectomy versus conventional four-port laparoscopic cholecystectomy. *Am J Surg* 2011; **202**: 254-258 [PMID: 21871979 DOI: 10.1016/j.amjsurg.2010.12.009]

30 **Pedraza R**, Aminian A, Nieto J, Faraj C, Pickron TB, Haas EM. Single-incision laparoscopic colectomy for cancer: short-term outcomes and comparative analysis. *Minim Invasive Surg* 2013; **2013**: 283438 [PMID: 23766897 DOI: 10.1155/2013/283438]

31 **Kang J**, Bae BN, Gwak G, Park I, Cho H, Yang K, Kim KW, Han S, Kim HJ, Kim YD. Comparative study of a single-incision laparoscopic and a conventional laparoscopic appendectomy for the treatment of acute appendicitis. *J Korean Soc Coloproctol* 2012; **28**: 304-308 [PMID: 23346509 DOI: 10.3393/jksc.2012.28.6.304]

32 **Aron M**, Berger AK, Stein RJ, Kamoi K, Brandina R, Canes D, Sotelo R, Desai MM, Gill IS. Transvaginal nephrectomy with a multichannel laparoscopic port: a cadaver study. *BJU Int* 2009; **103**: 1537-1541 [PMID: 19489791 DOI: 10.1111/j.1464-410X.2009.08612.x]

33 **Awad ZT**, Qureshi I, Seibel B, Sharma S, Dobbertien MA. Laparoscopic right hemicolectomy with transvaginal colon extraction using a laparoscopic posterior colpotomy: a 2-year series from a single institution. *Surg Laparosc Endosc Percutan Tech* 2011; **21**: 403-408 [PMID: 22146161 DOI: 10.1097/SLE.0b013e31823945ac]

34 **Benhidjeb T**, Stark M. An innovative technique for colorectal specimen retrieval: a new era of "Natural Orifice Specimen Extraction" (N.O.S.E.). *Dis Colon Rectum* 2010; **53**: 502-503; author reply 503 [PMID: 20305453 DOI: 10.1007/DCR.0b013e3181ca7dd7]

35 **Diana M**, Perretta S, Wall J, Costantino FA, Leroy J, Demartines N, Marescaux J. Transvaginal specimen extraction in colorectal surgery: current state of the art. *Colorectal Dis* 2011; **13**: e104-e111 [PMID: 21564461 DOI: 10.1111/j.1463-1318.2011.02599.x]

36 **Faller E**, Albornoz J, Messori P, Leroy J, Wattiez A. A new technique of laparoscopic intracorporeal anastomosis for transrectal bowel resection with transvaginal specimen extraction. *J Minim Invasive Gynecol* 2013; **20**: 333 [PMID: 23659753 DOI: 10.1016/j.jmig.2013.02.017]

37 **Fan JK**, Tong DK, Law S, Law WL. Transvaginal cholecystectomy with endoscopic submucosal dissection instruments and single-channel endoscope: a survival study in porcine model. *Surg Laparosc Endosc Percutan Tech* 2009; **19**: 29-33 [PMID: 19238063 DOI: 10.1097/SLE.0b013e3181902ba7]

38 **Fuchs KH**, Breithaupt W, Varga G, Schulz T, Reinisch A, Josipovic N. Transanal hybrid colon resection: from laparoscopy to NOTES. *Surg Endosc* 2013; **27**: 746-752 [PMID: 23052514 DOI: 10.1007/s00464-012-2534-7]

39 **Gaujoux S**, Bretagnol F, Au J, Ferron M, Panis Y. Single port access proctectomy with total mesorectal excision and intersphincteric resection with a primary transanal approach. *Colorectal Dis* 2011; **13**: e305-e307 [PMID: 21689345 DOI: 10.1111/j.1463-1318.2011.02676.x]

40 **Hara M**, Takayama S, Sato M, Imafuji H, Takahashi H, Takeyama H. Laparoscopic anterior resection for colorectal cancer without minilaparotomy using transanal bowel reversing retrieval. *Surg Laparosc Endosc Percutan Tech* 2011; **21**: e235-e238 [PMID: 22002283 DOI: 10.1097/SLE.0b013e3182297667]

41 **Wolthuis AM**, Penninckx F, D'Hoore A. Laparoscopic sigmoid resection with transrectal specimen extraction has a good short-term outcome. *Surg Endosc* 2011; **25**: 2034-2038 [PMID: 21136110 DOI: 10.1007/s00464-010-1472-5]

42 **Khoo RE**. Transanal excision of a rectal adenoma using single-access laparoscopic port. *Dis Colon Rectum* 2010; **53**: 1078-1079 [PMID: 20551763 DOI: 10.1007/DCR.0b013e3181ddf589]

43 **Léonard D**, Colin JF, Remue C, Jamart J, Kartheuser A. Transanal endoscopic microsurgery: long-term experience, indication expansion, and technical improvements. *Surg Endosc* 2012; **26**: 312-322 [PMID: 21898025 DOI: 10.1007/s00464-011-1869-9]

44 **Lorenz C**, Nimmesgern T, Back M, Langwieler TE. Transanal single port microsurgery (TSPM) as a modified technique of transanal endoscopic microsurgery (TEM). *Surg Innov* 2010; **17**: 160-163 [PMID: 20504794 DOI: 10.1177/1553350610370751]

45 **Choi BJ**, Lee SC, Kang WK. Single-port laparoscopic total mesorectal excision with transanal resection (transabdominal transanal resection) for low rectal cancer: initial experience with 22 cases. *Int J Surg* 2013; **11**: 858-863 [PMID: 23962662 DOI: 10.1016/j.ijsu.2013.08.003]

46 **Sylla P**, Rattner DW, Delgado S, Lacy AM. NOTES transanal rectal cancer resection using transanal endoscopic microsurgery and laparoscopic assistance. *Surg Endosc* 2010; **24**: 1205-1210 [PMID: 20186432 DOI: 10.1007/s00464-010-0965-6]

47 **Tuech JJ**, Bridoux V, Kianifard B, Schwarz L, Tsilividis B, Huet E, Michot F. Natural orifice total mesorectal excision using transanal port and laparoscopic assistance. *Eur J Surg Oncol* 2011; **37**: 334-335 [PMID: 21266304 DOI: 10.1016/j.ejso.2010.12.016]

48 **Zhou JN**, Wang DZ, Huang XE, Xu FP, Shang JQ, Gu RM. Transabdominal transanal resection of distal rectal cancer after high dose preoperative radiotherapy: a Chinese experience in preserving sphincter function. *Isr Med Assoc J* 2006; **8**: 675-678 [PMID: 17125111]

49 **Co CS**, Cheung HY, Yau KK, Chung CC, Li M. Combined single-port and endoluminal technique for laparoscopic anterior resection. *Surg Laparosc Endosc Percutan Tech* 2010; **20**: 253-256 [PMID: 20729696 DOI: 10.1097/SLE.0b013e3181e21b33]

50 **Hompes R**, Mortensen N, Cahill RA. Transanal endoscopic surgery using single access and standard laparoscopic instrumentation. *Minerva Gastroenterol Dietol* 2012; **58**: 273-281 [PMID: 22971637]

51 **Ragupathi M**, Ramos-Valadez DI, Pedraza R, Haas EM. Robotic-assisted single-incision laparoscopic partial cecectomy. *Int J Med Robot* 2010; **6**: 362-367 [PMID: 20665713 DOI: 10.1002/rcs.346]

52 **Choi GS**, Park IJ, Kang BM, Lim KH, Jun SH. A novel approach of robotic-assisted anterior resection with transanal or transvaginal retrieval of the specimen for colorectal cancer. *Surg Endosc* 2009; **23**: 2831-2835 [PMID: 19440794 DOI: 10.1007/s00464-009-0484-5]

P- Reviewers: Altomare DF, Bridoux V, Ruffolo C
S- Editor: Ma YJ **L- Editor**: A **E- Editor**: Liu XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.1005

World J Gastroenterol 2014 January 28; 20(4): 1005-1020
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Neuroanatomy of lower gastrointestinal pain disorders

Wim Vermeulen, Joris G De Man, Paul A Pelckmans, Benedicte Y De Winter

Wim Vermeulen, Joris G De Man, Paul A Pelckmans, Benedicte Y De Winter, Laboratory of Experimental Medicine and Pediatrics, Division of Gastroenterology, Faculty of Medicine and Health Sciences, University of Antwerp, 2610 Antwerp, Belgium
Paul A Pelckmans, Division of Gastroenterology and Hepatology, Antwerp University Hospital, 2650 Antwerp, Belgium
Author contributions: Vermeulen W reviewed the literature, designed and wrote the manuscript; De Man JG, Pelckmans PA and De Winter BY revised the manuscript critically and approved the final version.
Correspondence to: Benedicte Y De Winter, MD, PhD, Executive Director, Laboratory of Experimental Medicine and Pediatrics, Division of Gastroenterology, Faculty of Medicine and Health Sciences, University of Antwerp, Universiteitsplein 1, 2610 Antwerp, Belgium. benedicte.dewinter@uantwerpen.be
Telephone: +32-3-2652710 Fax: +32-3-2652567
Received: September 18, 2013 Revised: December 11, 2013
Accepted: January 6, 2014
Published online: January 28, 2014

Abstract

Chronic abdominal pain accompanying intestinal inflammation emerges from the hyperresponsiveness of neuronal, immune and endocrine signaling pathways within the intestines, the peripheral and the central nervous system. In this article we review how the sensory nerve information from the healthy and the hypersensitive bowel is encoded and conveyed to the brain. The gut milieu is continuously monitored by intrinsic enteric afferents, and an extrinsic nervous network comprising vagal, pelvic and splanchnic afferents. The extrinsic afferents convey gut stimuli to second order neurons within the superficial spinal cord layers. These neurons cross the white commissure and ascend in the anterolateral quadrant and in the ipsilateral dorsal column of the dorsal horn to higher brain centers, mostly subserving regulatory functions. Within the supraspinal regions and the brainstem, pathways descend to modulate the sensory input. Because of this multiple level control, only a small proportion of gut signals actually reaches the level of consciousness to induce sensation or pain. In inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) patients, however, long-term neuroplastic changes have occurred in the brain-gut axis which results in chronic abdominal pain. This sensitization may be driven on the one hand by peripheral mechanisms within the intestinal wall which encompasses an interplay between immunocytes, enterochromaffin cells, resident macrophages, neurons and smooth muscles. On the other hand, neuronal synaptic changes along with increased neurotransmitter release in the spinal cord and brain leads to a state of central wind-up. Also life factors such as but not limited to inflammation and stress contribute to hypersensitivity. All together, the degree to which each of these mechanisms contribute to hypersensitivity in IBD and IBS might be disease- and even patient-dependent. Mapping of sensitization throughout animal and human studies may significantly improve our understanding of sensitization in IBD and IBS. On the long run, this knowledge can be put forward in potential therapeutic targets for abdominal pain in these conditions.

Key words: Afferent nerves; Chronic pain; Inflammatory bowel disease; Irritable bowel syndrome; Sensitisation; Sensory nerves; Visceral hypersensitivity

Core tip: This review reports on the neuroanatomy of gastrointestinal pain disorders. The encoding and conveying of information from the gastrointestinal wall to the central nervous system is described with emphasis on the peripheral level, the spinal cord and the higher brain centers. Besides the basic principles of visceral hypersensitivity are reviewed taking into consideration peripheral sensitization (and the main mediators involved) and central wind-up phenomena in order to better understand the mechanisms involved in chronic abdominal pain in inflammatory bowel disease and irritable bowel syndrome patients.

Vermeulen W, De Man JG, Pelckmans PA, De Winter BY. Neuroanatomy of lower gastrointestinal pain disorders. *World J Gastroenterol* 2014; 20(4): 1005-1020 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/1005.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.1005

INTRODUCTION

Chronic abdominal pain frequently originates from a local inflammatory reaction in the lower gastrointestinal (GI) tract. Indeed, 50%-70% of patients with inflammatory bowel disease (IBD), encompassing ulcerative colitis (UC) and Crohn's disease (CD), present with abdominal discomfort or pain[1,2]. The bulk of IBD research merely focuses on treatment algorithms for the achievement and the maintenance of endoscopic remission of mucosal lesions but this approach insufficiently resolves concomitant pain[3,4]. A retrospective 5 year follow-up study in CD patients revealed a high prevalence of chronic narcotic users which exemplifies the analgesic shortcoming[5]. Farrokhyar and colleagues reported symptoms compliant to Rome Ⅱ diagnostic criteria for irritable bowel syndrome (IBS) in approximately one-third of patients with inactive CD[4,6]. IBS entails a heterogeneous group of functional lower GI disorders characterized by abdominal pain and altered bowel habits[7]. While routine diagnostic tests exclude alarming features for organic pathology, some though not all investigators discovered subclinical evidence of a low-grade ileitis and colitis in several IBS patients such as mildly elevated levels of fecal calprotectin[8,9], an increase in proinflammatory cytokines including IL-6, IL-8, tumor necrosis factor (TNF)-α and IL-1β[10], colonic lymphocytosis and mastocytosis[11]. Aside from concerns of some regarding these findings[12,13], observations linking symptoms to a bout of enteritis hypothesize that a previous transient inflammation contributes to IBS etiology[14]. IBD on the other hand manifests as a chronic uncontrolled immunologic reaction with recurrent flares of inflammation[15]. Hence, there are indications supporting and opposing overlap between IBD and IBS. A more acknowledged statement, regardless of the inflammatory origin, is that the pathology has imprinted changes in the gut-brain neuronal connection and so manifests in a diffuse and poorly localized chronic pain in the abdomen. The aforementioned features characterize pain in IBS and IBD patients and are partially attributed to the complex sensory innervation pattern of the pelvis. The colorectum is extensively innervated, albeit only a low density of extrinsic afferents provides the substantial link with the central nervous system (CNS) for perception. The extrinsic colon afferents branch to other organs (*e.g.*, colorectum, bladder, reproductive organs) within the abdominal cavity and organize into web-like plexuses scattered through the abdomen. Due to this anatomical organization signals of multiple pelvic organs, rather than restricted to the colon, converge to a relatively extensive number of spinal cord segments[16]. Within the spine other body signals may join and together the information travels *via* supraspinal levels to the higher brain centers. Importantly it is known that somatic input outnumbers the visceral input and that the visceral input is entering the spinal cord at different segments. This ambiguous signal transduction from the gut *via* the three-neuron chain to the brain explains why the experienced pain has a poor topological relation and cannot always be pinpointed. In IBD and IBS, each level of control is susceptible to neuromodulatory changes or sensitization. Therefore, we review how stimuli in the healthy and the hypersensitive bowel are detected, encoded, and conveyed to the brain to be either unconsciously, consciously or painfully perceived. For reasons of clarity, we will start with a description of the anatomical structures involved.

PERIPHERAL AFFERENT PATHWAYS SUPPLYING THE GASTROINTESTINAL TRACT

Intrinsic innervation: the enteric nervous system

The intestinal wall of the esophagus, the stomach, the small intestine and the colon house a dense neuronal network (about 10^8 neurons), the enteric nervous system (ENS), also referred to as the "little brain-of-the-gut". This intrinsic network comprises enteric nerve cell bodies of sensory, inter- and motor neurons grouped into ganglia and interconnected by bundles of nerve processes forming plexuses of which the best characterised are the myenteric plexus (Auerbach's plexus) and the submucosal plexus (Meissner's plexus)[17-19]. The ENS controls motility, mucosal secretion and absorption, mucosal growth, local blood flow and the immune function in the gut[18]. The connective link between the CNS and the ENS is bidirectional: the brain influences the function of the ENS and *vice versa*. When the brain encounters stressful life events, the lower gut gets overstimulated resulting in diarrhea. When the lower gut responds to food poisoning with powerful propulsive colon contractions, the body experiences aversion towards the ingested meal and abdominal cramping pain. With referral to the latter, high-amplitude propagating contractions in the ileocecum and sigmoid colon of IBS patients in response to eating correlate to abdominal pain[20]. Support to this hypothesis comes from reports on antispasmodics giving short-term pain relief in at least a subset of diarrhea-predominant IBS patients[21]. Likewise, antispasmodic agents may be effective in IBD, especially in those patients who are in remission and have mild to moderate chronic pain[22]. Besides their role in ileocolonic dysmotility, intrinsic enteric afferents containing serotonin, substance P, CGRP can initiate or intensify neurogenic inflammation upon release and thereby sensitise adjacent extrinsic gut nerves. The relevance of the enteric nervous system to pain primarily lies within the excitation of these extrinsic afferents by neuropeptides.

Extrinsic sensory innervation of the gastrointestinal tract

The extrinsic primary afferents of the GI tract provide the anatomical connection with the CNS and so a basis for both nonpainful (*e.g.*, satiety, passage of gas, *etc.*) and painful (*e.g.*, inflammation, ischemia, extensive distension) gut sensations. The GI tract receives a dual innervation with complementary roles in gut signaling: a splanchnic and a vagal plus pelvic afferent population. These afferents run alongside the efferent orthosympathetic (splanchnic nerves) and parasympathetic nervous system (vagal/pelvic nerves) respectively, but are never referred to as such[23]. It is assumed that the vagal/pelvic nerves subserve homeostatic functions, whereas the splanchnic innervation principally conveys nociception. This simple dichotomy of function, however, appears far more complex than formerly assumed.

Vagal innervation: The vagal nerve is the largest sensory pathway in the body with up to 80% of the fibers being afferents. The vagal nerve branches to the entire gut, except the transverse and distal portion of the colon. The vagal cell bodies reside in the ganglion nodosum and the central nerve endings terminate in the nucleus of the solitary tract in the dorsal medulla. Vagal afferents mainly regulate feeding behavior by upper gut reflexes (*e.g.*, gastric accommodation, gastric emptying, gastric/pancreatic secretion, emesis) and the perception of hunger, fullness, satisfaction, bloating and nausea. Three types of vagal fibers were characterized in the mouse by an *in vitro* vagus-gastro-esophageal set-up: mechanoreceptors, tension receptors and specific chemoreceptors activated by bile. Vagal mechanical afferent endings within the muscle layers are classified into two types: intramuscular arrays and intraganglionic laminar endings. Intramuscular arrays run parallel in either the longitudinal or circular muscle layers and have been suggested to respond to muscle stretch. Intraganglionic laminar endings branch extensively in connective tissue surrounding myenteric ganglia and convey info about distension and muscle contraction[24]. The tension-sensitive afferents respond maximally at distensions within the physiological range and are activated by normal peristaltic contractions. This implies that the vagal afferents take care of physiological perception of mechanical stimuli[25,26], whereas pain evoked by distension of the upper GI tract is probably mediated *via* the splanchnic afferents. However, vagal afferents have been shown to be implicated in the pain reactions evoked by gastric acid challenge[27]. The activity of lower gut vagal afferents modulates spinal transmission. The latter is supported by the observation of increased pain responses to colorectal distension after subdiaphragmatic vagotomy in rats[28,29]. In humans, lower thresholds for the perception of pain have been described in patients who had previously undergone vagotomy in the course of a Billroth I gastrectomy compared with pain thresholds in healthy controls[30]. The exact mechanism is still controversial, but likely involves specific relay nuclei such as the nucleus raphe magnus and ventral locus coeruleus. On the one hand vagal afferents have been shown to facilitate nociceptive transmission[25], whereas on the other hand the vagal nerve appears to participate in an antinociceptive descending pathway mediated by nanomolecules such as but not limited to opioids[28,31]. This discrepancy may be explained by differences in stimulation parameters: low intensity stimulation of vagal afferents facilitates, while high intensity stimulation inhibits nociception[31]. IBD patients may benefit from chronic vagal stimulation since the vagal nerve stimulation exerts anti-inflammatory effects. Previous studies in a sepsis model showed that the vagal nerve regulates the cholinergic tonus so that the immune response of macrophages and immunocytes is dampened[32].

Pelvic innervation: The pelvic nerves mainly innervate pelvic structures: the colorectum, the bladder and the reproductive organs. Pelvic afferents represent 30%-50% of the total number of neurons and converge visceral information onto spinal neurons in the lumbosacral segments L6-S2 of the spinal cord in mice and rats[33]. Their cell bodies are located in the lumbosacral dorsal root ganglia (DRG). The pelvic nerve contains serosal, mucosal, muscular (*e.g.*, intraganglionic laminar ending and intramuscular arrays), muscular/mucosal afferents and is specialized to detect circular stretch, the primary stimulus generated by low-intensity colorectal distension or stool passage[34]. Pelvic afferents transmit similar modalities of information as the vagal afferent system *i.e.,* physiological sensation (*e.g.*, urgency, desire to defecate, *etc.*). In addition, animal data support the idea that they form the afferent branch of extrinsic gut reflexes such as the cologastric inhibitory reflex[35,36]. They are a subject of interest in pain research as a bilateral pelvic nerve section almost entirely abolished pain-related behavior to noxious colorectal distension in rats[23,34]. Following TNBS colitis, however, pain responses partially recovered[37]. Hence, the pelvic nerve is involved in normal physiology and acute pain, rather than in inflammatory pain which is more specifically mediated by splanchnic afferents. However, animal experiments in rat have shown pelvic fiber sensitization when a chemical irritant is applied to colonic tissue, posing a role in nociception of the pelvic nerve under inflammatory circumstances[36].

Splanchnic innervations: The splanchnic nerves innervate the entire GI tract and are the functional counterpart of the vagal plus pelvic nerves. Visceral afferents located in splanchnic nerves project to the spinal cord. Unlike the pelvic afferents, visceral information from the colorectum carried by the splanchnic nerves project onto thoracolumbar segments T10 - L2 in mice and rats[33]. Their cell bodies are located in the thoracolumbar DRG near the spinal cord, with peripheral projections ending at various levels within the gut wall. These splanchnic afferent fibers course through the prevertebral ganglia (celiac, superior and inferior mesenteric ganglion) where

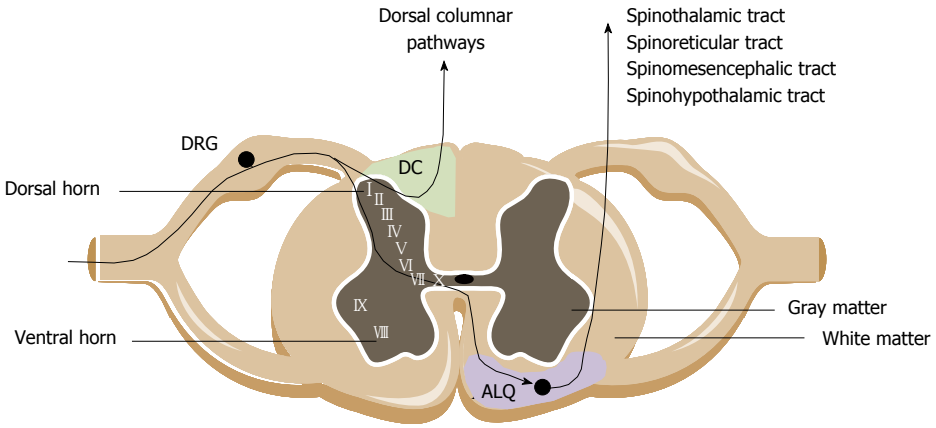


Figure 1 Cross section of the spinal cord. The central branches of the visceral afferents innervating the lower gastrointestinal tract travel *via* the dorsal root ganglia (DRG) and project onto the second order neurons in laminae Ⅰ, Ⅱ, Ⅴ, Ⅹ of the spinal gray matter. Ascending pathways arise in the anterolateral quadrant (ALQ; purple zone) and the dorsal column (DC; green zone) region in the spinal cord and project to higher brain centers (*e.g.*, medulla, thalamus).

they may form "en passant" synapses with efferent sympathic neurons. From animal data splanchnic afferents are thought to constitute the main nociceptive pathway from the gut as they signal different modalities of mechanosensory information[34,38]. In the upper GI tract splanchnic afferents appear to mediate gastric mechano-nociception, but not gastric chemo-nociception which is mediated by vagal sensory neurons. This is supported by the finding that in rodents pain-related responses to gastric distension are blocked by splanchnicectomy, but not by vagotomy[39]. In the lower GI tract, splanchnic afferents convey signals of abdominal discomfort. Indeed, the majority of splanchnic afferents of the colon encountered in mice are located in the serosa (36%) and mesenteric (50%) membranes, often associated with mesenteric blood vessels. Likewise, the mucosal spinal afferents are often found near submucosal blood vessels where they form varicose branching axons. The bare endings of the submucosa and mesenteric afferents are likely to respond to distortion of the gut during stretch or contraction, especially at levels that give rise to pain[40,41]. Hence, they are tuned to encode stimuli into the noxious range[34]. In the rat, both anatomical and functional evidence points to a specific role of splanchnic afferents in pain during colitis[37,42].

Very recently Brookes and co-authors suggest a novel classification into five "structurally distinct" types of sensory endings within the gut wall forming the anatomical extrinsic sensory pathways described by these authors as the vagal pathway, the thoracolumbar spinal pathway projecting *via* the splanchnic nerves and the lumbosacral spinal pathway projecting *via* the pelvic and rectal nerves[23].

CENTRAL PATHWAYS CONVEYING VISCERAL SIGNALS FROM THE LOWER GI TRACT

Extrinsic primary afferents innervating the lower GI tract primarily synapse with second-order neurons in the dorsal horn of the thoracolumbar and lumbosacral spinal cord segment. Fibers terminate predominantly in the superficial laminae Ⅰ and Ⅱ, but also reach deeper layers such as the laminae Ⅴ and Ⅹ of the gray matter. Ascending pathways project to higher brain centers where pathways origin and descend to fine-tune the sensory input[43,44].

Ascending pathways

Central ascending pathways involved in bowel sensations include both pathways ascending in the anterolateral quadrant (ALQ) of the white commissure and the dorsal column of the dorsal horn (Figure 1). Pathways ascending in the *ALQ* transmit noxious cutaneous stimuli and also carry nociceptive information of visceral origin. This idea is largely based on anterolateral cordotomies performed in the 20th century to relieve pain due to damage to the spinal cord by disease or trauma[45]. The pathways in the ALQ are the spinoreticular, spinomesencephalic, spinohypothalamic and spinothalamic tracts[46]. The former three tracts mainly subserve regulatory functions below the level of consciousness. The spinoreticular tract projects to the dorsal reticular nucleus in the brainstem, which is involved in the affective-motivational properties (emotional component of pain) of visceral stimulation. The spinomesencephalic tract conveys information from the spinal cord to the periaqueductal gray (PAG) and other midbrain regions. The spinohypothalamic tract conducts sensory information from the spinal cord directly to the hypothalamus. The hypothalamus together with other parts of the limbic system (amygdala, medial thalamus, ACC), locus coeruleus and PAG regulate arousal and emotional, autonomic and behavioral responses. The spinothalamic tract mediates the sensations of pain, cold and heat, and also contributes to touch sensation. Projections of the spinothalamic tract have been traced to the thalamus in humans and in laboratory animals. The thalamus is a major relay station where multiple somatic and visceral inputs converge. Before the information is conveyed *via* the third order neurons to the cortex, the thalamus will process the nociceptive information. Human observations coupled with an extensive repertoire of experimental data suggest that particularly the posterolateral nucleus of the thalamus is involved in the processing of visceral information, including both innocuous and noxious visceral inputs. The thalamus, relays to cortical regions such as the pregenual anterior cingulate cortex (pACC), mid cingulate cortex, the insula and the somato-

sensory cortex. Notably, visceral sensation is primarily represented in the secondary somatosensory cortex[47]. In these cortical regions the nociceptive signals are processed, integrated and eventually perceived as "painful". Brain images provided by H(2)(15)O micro positron-emission tomography (PET) scanning performed during colorectal distension in rats suggest that the cerebellum is also involved in visceral nociception[48], which is supported by findings in healthy humans documenting cerebellar activation in response to painful visceral stimuli such as distension of the colorectum[49]. A number of recent studies has pointed to a specific role of the *dorsal funiculus* [*dorsal column* (DC) in animals] in viscerosensory transmission and visceral nociception. Experimental data from different groups have identified the DC as being more important in visceral nociceptive transmission than the spinothalamic, spinohypothalamic, spinomesencephalic and spinoreticular tracts[50,51]. The bulk of evidence rests on the great effectiveness of limited midline myelotomy in reducing intractable pelvic cancer-related pain in humans and on a number of experimental observations in animals[52]. The DC contains collateral branches of primary afferent fibers that ascend from the dorsal root entry level to the medulla. In addition, it contains the ascending axons of tract cells of the dorsal horn. These tract cells form the postsynaptic DC pathway, which along with primary afferent axons, travel in the DC and synapses in the DC nuclei. The postsynaptic DC cells in rats and monkeys were shown to receive inputs from the colon, the ureter, the pancreas and epigastric structures[53]. A DC lesion does not reduce pain caused by noxious cutaneous stimuli in humans[54], which argues for a selective role of the DC pathway in visceral pain such as pain evoked by enteritis.

Descending pathways

It is well recognized that spinal nociceptive transmission is modulated by descending pathways from various supraspinal structures, including the nucleus raphe magnus, the periventricular gray of the hypothalamus and the midbrain PAG. At cortical level, the ACC is the most important source of descending modulatory pathways, projecting to the amygdala and the PAG which is probably the key pain modulatory region. Descending modulation of spinal nociceptive processing can be either inhibitory or facilitatory. In the late 1960s it was shown that focal electrical stimulation in the midbrain PAG of the rat permitted abdominal surgery in the absence of general anesthesia due to the pain suppressive effects of stimulation of this specific region[55]. The PAG - rostral ventromedial medulla (RVM) - dorsal horn circuitry is the best characterized nociceptive modulatory pathway through which pain is endogenously inhibited. Endogenous opioids are key mediators in the descending pain inhibitory pathways. Specifically the pACC is assumed to send inhibitory signals to pontomedullary networks since it contains a high content of opioids. Additionally, monoaminergic neurotransmitters such as noradrenaline, serotonin and dopamine positively or negatively modulate pain signaling with remarkably opposing effects, depending on the extent of transmitter release, the receptor type, receptor affinity and the location in the spinal cord the descending pathways project towards[47,56,57]. Further, it is shown that the excitability of spinal dorsal horn neurons to peripheral sensory stimulation are enhanced or increased by stimulation of the RVM including the reticular formation of the serotonergic nucleus raphe magnus[58-60]. These findings support a role of the RVM and raphe magnus in a facilitatory descending pathway.

PRINCIPLES OF VISCERAL HYPERSENSITIVITY

Visceral hypersensitivity refers to an increased perception of stimuli arising from the viscera. Specific terms are used to describe the hypersensitivity: *allodynia* and *hyperalgesia*. The perception of pain in response to stimuli that are normally not perceived as painful is referred to as allodynia. The term allodynia strictly does not apply to visceral pain since the visceral organs are normally almost insensate but the concept of visceral allodynia is useful to understand sensitization in a variety of gut disorders. An increase in pain perception to stimuli that are normally perceived as painful is referred to as hyperalgesia[61]. Concerning IBD and IBS, we focus this review on colorectal hypersensitivity. A hypersensitive colorectum is considered the hallmark feature of all IBS subtypes[62,63] as altered rectal perception is documented in 61% of IBS patients meeting Rome Ⅱ criteria[64]. It is currently the most widely accepted mechanism for abdominal pain. Some investigators have even suggested that this physiological hallmark is useful in clinical diagnosis[65]. Based on the current scientific evidence, the mechanisms of visceral hypersensitivity have been formulated in a number of hypotheses. These include (1) the sensitization of peripheral visceral afferent neurons; (2) the sensitization of spinal cord dorsal horn neurons; (3) the altered descending excitatory and inhibitory influences to the spinal cord nociceptive neurons; and (4) the misinterpretation of innocuous sensation as noxious due to cognitive and emotional biasing (*e.g.*, hypervigilance, pain catastrophizing)[47,66]. The degree to which each of these mechanisms generate visceral hypersensitivity and therefore pain symptoms is still unclear. However, it is assumed that these mechanisms are rather complementary than mutually exclusive.

Peripheral sensitization

The gut is not only provided with an extensive neuronal network, it also houses highly specialized immunocytes and epithelial cells equipped with the machinery to participate in sensitization in the event of a potential threat[67]. In IBD and some IBS subsets, inflammation likely triggers the peripheral sensitization. Enterochromaffin cells (ECC) and mast cells function as intermediaries between the "inflammatory soup" (*e.g.*, tissue

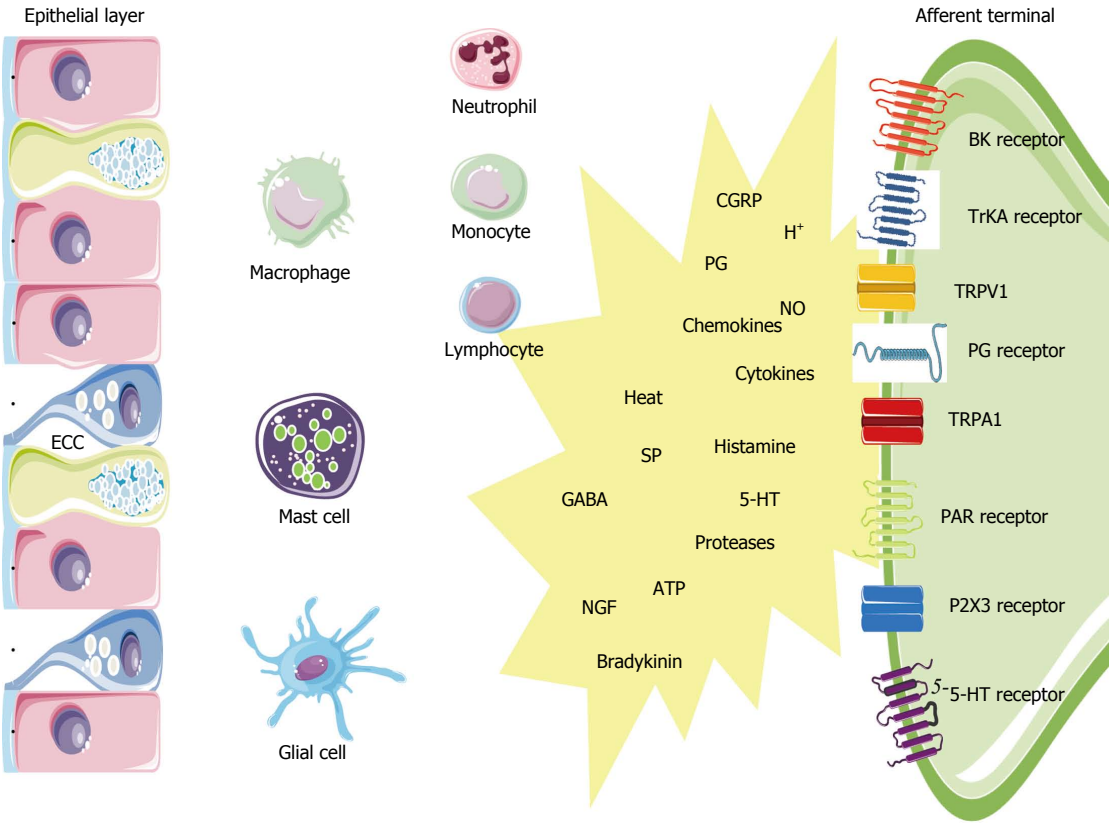


Figure 2 Scheme is oversimplified and limited to the cell types and mediators discussed in this review and represents a subset of cells and inflammatory mediators responsible for activation of gut sensory afferents after an initial inflammatory response. 5-HT: 5-hydroxytryptamine; BK: Bradykinin; CGRP: Calcitonin-gene-related peptide; ECC: Enterochromaffin cell; GABA: Gamma-amino butyric acid; NGF: Nerve growth factor; NO: Nitric oxide; PAR: Proteinase-activated receptor; PG: Prostaglandin; SP: Substance P; TrKA: Tyrosine receptor kinase A; TRPA1: Transient receptor potential ankyrin-1; TRPV1: Transient receptor potential vanilloid-1; P2X3: Purinergic P2X3 receptor.

acidosis, cytokines, arachidonic acid metabolites) and the neuroenteric system (Figure 2). ECC are interposed between epithelial cells of the GI mucosa where they act as sensors or "taste bottoms" of the intraluminal milieu. EEC contain large numbers of electron-dense secretory granules with a range of peptides such as but not limited to serotonin, cholecystokinin and secretin positioned towards their basement membrane. In response to luminal nutrients, toxins and mechanical stimulation the ECC release their content into the gut wall which influences the neuromuscular apparatus. Serotonin release for instance is well known to activate vagal afferent endings in the upper GI tract serving as an emetic trigger[68]. A proportion of postinfectious irritable bowel syndrome (PI-IBS) patients have ECC hyperplasia and multivariate analysis has shown that ECC count is an important predictor of developing PI-IBS (relative risk 3.8)[4,69]. Also the endocrine cell population in patients with CD ileitis showed an increase in ECC number, both at affected and nonaffected sites of the ileum. In a study on colonic tissue, the ECC area was likewise significantly increased in active CD and UC[47]. The same was found in colorectal tissue from UC patients in remission. Recently, a nematode-infected (Trichuris muris) immunodeficient mice model revealed an interaction between $CD4^+$ T cells and ECC. The infection evoked Th2 response lead to ECC hyperplasia *via* the presence of IL-13 receptors on ECC, resulting in an increase in serotonin production[70]. The 5-HT receptor subtypes that are involved in visceral hypersensitivity are 5-HT_3, 5-HT_4 and 5-HT_{2B}. 5-HT_3 antagonists (alosetron and cilansetron) prevent the activation of 5-HT_3 receptors on extrinsic afferent neurons and decrease hyperalgesia and abdominal pain in IBS patients[71]. More recently, evidence emerged that 5-HT_4 receptor-mediated mechanisms regulate visceral sensitivity as tegaserod, a partial 5-HT_4 agonist, normalized postinflammatory hypersensitive colon in the rat[72]. In a recent patient study, tegaserod significantly reduced the inhibitory effects of colorectal distension on the RIII reflex in 12 of 15 patients[73]. Finally a role for 5-HT_{2B} has been stated, but needs further verification. Serotonergic mechanisms are likely implicated in PI-IBS patients based on an increased number of ECC[74-76], an increased mast cell population[77], an increased postprandial serotonin release[78]. The metabolism of 5-HT might also be disrupted in both IBS and IBD. In this regard, it has been suggested that decreased serotonin-selective reuptake transporter (SERT) expression in IBD and IBS patients is associated with GI dysfunction in these disorders[79-81]. SERT, which is expressed on enterocytes, terminates the actions of serotonin by removing it from the interstitial space. The role of SERT in GI pathology is further sup-

ported by the observation that colonic sensitivity to CRD was attenuated in mice after long-term treatment with paroxetine, a SERT inhibitor[82]. Polymorphisms of the serotonin re-uptake transporter gene may also play a role in disturbance of gut function. IBS patients with deletion/deletion genotype of SERT polymorphism more often experience abdominal pain compared to those expressing other SERT polymorphisms[83].

Mast cells are bone-marrow derived cells that circulate in the bloodstream as immature progenitors and maturate and reside within the mucosal and connective tissues (Figure 2). Mast cells possess a plethora of mediators that can be rapidly released out of preformed granules like histamine, serotonin, serine proteases (*e.g.*, tryptase), proteoglycans or that can be *de novo* synthetized such as prostaglandins (*e.g.*, PGE_2, PGD_2), leukotrienes (*e.g.*, LTC_4, LTD_4), platelet activating factor (PAF), and cytokines (*e.g.*, TNFα, IL-6)[84,85]. In mice, serotonin is also present in mucosal mast cells in the lamina propria and some studies have suggested that human mast cells may also contain serotonin especially in conditions associated with mastocytosis[86]. Within the GI wall the close proximity between mast cells and neurons is intriguing and a bidirectional interaction between them is generally accepted[67,87,88]. Recently, it was shown that the number of mast cells close to afferent fibers was significantly increased in rats with DSS colitis, and that nerve fibers reacted stronger to compound 48/80-evoked mast cell degranulation[89]. Visceral hypersensitivity, evoked by the chemical irritant TNBS but also by chronic juvenile stress from maternal separation, can be treated with the mast cell stabilizer ketotifen and is abolished in mast cell deficient rats[90,91]. In patients, ketotifen increases the threshold for discomfort in patients with IBS with visceral hypersensitivity, reduces IBS symptoms and improves health-related quality of life regardless from the poor correlation with mast cell activation in biopsies[92]. There is considerable clinical evidence for mast cell involvement in human IBD. In colorectal mucosa from patients with CD and UC, the amount of mast cell tryptase was significantly increased, as was the number of mast cells in the lamina propria and submucosa[93]. The same observations were made in the afflicted ileum of CD patients[4,94]. The secretion profile of mast cells derived from UC patients was also shifted, releasing greater amounts of histamine, PGs and leukotrienes[95,96]. Moreover, rates of tryptase secretion were increased in both inflamed and noninflamed tissue from UC patients indicating that mast cell activation/proliferation can be altered remote from the site of active inflammation[4,97]. Also in IBS patients sufficient data support a role for mast cells. Mast cell infiltration was associated with symptoms of bloating in IBS patients[98]. In addition, activated mast cells in close proximity to colonic nerves were significantly correlated with the severity and frequency of abdominal pain or discomfort[99]. The experimental observation that mast cell mediators, released from the colonic mucosal biopsies from IBS patients but not of healthy patients, excite rat nociceptive visceral afferents further provide evidence of a pivotal role of mast cells in IBS hypersensitivity and have been confirmed by several other groups in different countries[100,101].

Protease-activated receptors (PARs) are a family of 4 receptor types activated by serine proteases such as thrombin and tryptase (Figure 2). PARs have a widespread distribution throughout the GI tract enabling the involvement in all the aspects of gut physiology, including inflammation and nociception. PAR1, 2 and 4 have been implicated in the modulation of nociceptive mechanisms, since they are expressed by nociceptive DRG neurons containing CGRP and SP. However, the function of these receptors in nociceptive signaling might be opposite. PAR2 was found to be activated by trypsin and the mast cell mediator tryptase, whereas PAR1 and 4 are activated by thrombin[102]. In rodents, PAR1 and PAR4 exert antinociceptive signals whereas PAR2 is clearly a pronociceptive agent regarding both mast cell- and formalin-induced hyperalgesia in mice[103]. A role for PARs in inflammatory disorders is evidenced by several studies: the expression of PAR1 and PAR2 is upregulated in tissues from CD or UC patients[104,105], the levels of the PAR2 agonists trypsin and mast cell tryptase are elevated in mouse colon[106], elevated colonic luminal serine protease activity has been observed in IBS-D patients[107]. The generation of pain symptoms has been suggested by the observation that mice injected with mediators released from colonic biopsies of IBS patients, exhibit enhanced nociceptive responses to CRD, whereas transgenic mice without PAR2 failed to show such mechanical hyperalgesia[107,108]. From these findings, it would appear that PAR2 antagonists and PAR1 and PAR4 agonists have potential in the control of visceral pain and hyperalgesia symptoms in both IBD and IBS. In mice, PAR2-mediated mechanical hyperalgesia requires sensitization of the ion channel transient receptor potential vanilloid 4 (TRPV4), since deletion of TRPV4 prevented PAR2 agonist-induced mechanical hyperalgesia and sensitization[109,110]. Accordingly, mast cell tryptase-induced PAR2 activation is proposed as a mechanism for TRPA1 sensitization as it was shown that PAR2-induced hyperalgesia was absent in TRPA1 knockout mice[111].

Nerve growth factor (NGF) is synthetized by epithelial cells and mast cells when triggered by IL-1β and TNFα (Figure 2)[112]. NGF influences development and function of afferents by binding to its high affinity TrKA receptor. Indeed, NGF can modulate the expression of membrane bound receptors such as TRPV1 and TRPA1 localized at peripheral afferents. The described NGF-mediated mechanism could regulate inflammatory hyperalgesia seen in IBD, as hypersensitivity in rats with inflamed colon can be reversed by anti-NGF antibody treatment[113]. NGF has been implicated in several chronic inflammatory processes. In CD, NGF mRNA is increased in 60% and TrkA mRNA in 54% in UC, NGF mRNA expression was enhanced in 58% (2.4-fold; $P < 0.01$) and TrkA mRNA expression in 50% of the patients. Enhanced expression of NGF and TrkA in both neural and non-neural structures suggests activation of

this neuroimmune pathway in chronic inflammation in CD and UC[114].

A population of cells that is recently taken into account in the modulation of neuroimmune interactions are the peripheral glial cells. These cells are capable of modulating enteric neurotransmission, modulate inflammation and control intestinal barrier function. They are capable of these interactions as they contain precursors for neurotransmitters such as GABA and NO; they express receptors for purines and they are able to produce cytokines (IL-1β, IL-6, TNFα), NGF and neuropeptides (NKA and SP) after activation[115]. There is recent evidence for a paracrine purinergic neuro-glial communication and also after injection of endotoxins in mice glial cells are activated[116,117]. Changes in enteric glial cells have been described in IBD[118]. Recently, the role of glial cells has been investigated in rectal biopsies of UC patients; the expression of S100, a marker for enteric glial cells, was associated with an increase of inducible nitric oxide synthase expression[119]. Inflammation increases the synthesis of PGs through upregulation of cyclooxygenase-2 (COX-2). For instance, in patients with active CD and UC a six- to eightfold increase in COX-2 mRNA was demonstrated in the bowel wall[120]. Although suppression of PG production in the gut by COX inhibitors carries the risk of severe GI mucosal damage, blockade of PG receptors expressed by sensory neurons appears to be an alternative way of preventing the proalgesic action of PGs. PGE_2 and PGI_2 have been proven to be key mediators of inflammatory hyperalgesia. Primary afferent neurons express PG receptors of the EP_1, EP_2, EP_3, EP_4 and IP type. PG receptors are also found at the central synapse in the spinal cord. For instance, PGE_2 is recognized as playing a prominent role in the CNS as well as peripheral tissues[121].

Perhaps the most intriguing players in peripheral hyperalgesia and pain are the transient receptor potential (TRP) ion channels of the vanilloid type 1 (TRPV1), the vanilloid type 4 (TRPV4), the ankyrin type 1 (TRPA1) and the melastatin type 8 (TRPM8) (Figure 2). These TRPs are expressed on gut afferents including those that conduct noxious stimuli to the spinal cord and co-express with the above listed G protein-coupled receptors (*e.g.*, EP1, $5HT_3$, BK1, PAR2) and growth factor receptors (*e.g.*, TrKA receptors). Their close proximity allows the TRPs to couple their activity to mutual downstream pathways which enables them to integrate a diversity of stimuli present in the inflammatory milieu[122]. TRPV1, the best characterized TRP, acts as an immediate sensory alarm in response to mechanical stretch or distension, mild acidification (pH < 5.9), noxious heat (> 42 ℃) and spice ingredients such as capsaicin[123,124]. Also endovanilloids such as anandamide, unsatured N-acyldopamines and lipoxygenases of arachidonic acid are known to directly activate TRPV1. Many proalgesic factors are associated with TRPV1-induced hyperalgesia (*e.g.*, bradykinin, 5-HT, NGF, PAR2, endogenous metabolites)[125]. Experimental animal models have shown that *TRPV*$^{-/-}$ mice have decreased pain-related responses to colorectal distension, whereas capsaicin application will increase pain to colorectal distension in animals. These data in animals suggest the involvement of TRPV1 signalling pathways in colonic pain. Of clinical relevance is that TPRV1 expression is increased in UC and CD[126]. TRPV1 is upregulated not only in inflammation but also in the absence of overt inflammation as is typical of functional GI disorders. This is true for patients with IBS in which increased density of TRPV1 in the rectosigmoid correlated with pain severity[127]. A similar correlation between pain intensity and number of mucosal TRPV1-positive nerve fibers is found in patients with quiescent IBD who continue to complain of abdominal pain[126,127]. TRPA1 is a receptor characterized by a long ankyrin repeat at the N-terminal site that serves as a binding site for a range of environmental irritants, oxidants and spices such as mustard oil, wasabi and horseradish[128]. Recent findings have also found that TRPA1 is involved in cold transduction and mechanosensation[129,130]. Moreover, TRPA1 contributes to inflammatory hyperalgesia *via* PAR2 activation[111]. Clinically, an autosomal dominant mutation in the fourth transmembrane of TRPA1 was described in one family that underlies a familial episodic pain syndrome[131]. Further, TRPA1 is a candidate mechanosensor for mechanical hyperalgesia in colitis and overactive bladder[130,132]. TRPA1 is almost exclusively present in a TPRV1-positive population of sensory nociceptors and does not co-express with TRPV1 in other tissues. In this regard, a study quantifying TRPA1-positive neurons in trigeminal ganglia has demonstrated that TRPA1 is expressed in anout 55% of TRPV1-positive neurons while NGF treatment of trigeminal ganglia increases TRPA1 expression to anout 80% of TRPV1-positive cells. Several lines of evidence has shown that TRPV1 exerts a modulatory role on TRPA1 channels[133]. With concern to hypersensitivity of the colon, we recently have shown that TRPV1 and TRPA1 synergistically decrease viscero-motor responses in rats with TNBS colitis but not in control rats[134].

Central sensitization

Apart from sensitization in the periphery, the gut impulses are modulated or amplified in the spinal cord and higher brain centers; a process referred to as central sensitization. The co-morbidity of IBS with disorders such as but not limited to depression, anxiety, and painful bladder syndrome or of IBD with interstitial cystitis may originate from central sensitization[16,135,136]. The leading hypothesis to explain these co-occurrences is a viscerovisceral and a viscerosomatic cross-sensitization, with somatic and visceral afferents converging onto the same second order neuron in the spinal cord or third order neuron in the supraspinal centers and an overlap within yet undefined brain fields[137]. Clinical evidence for a role of CNS sensitization in visceral pain comes from functional resonance magnetic imaging (fMRI) and PET studies on referred pain to adjacent structures or at

remote distance from the (actual) injured organ[138]. More direct evidence for enhanced spinal processing in IBS patients has been confirmed through analysis of rectal distensions on the RIII reflex, a nociceptive withdrawal reflex used as an objective tool to investigate pain processing at the spinal and supraspinal level. Whereas slow ramp rectal distension induced inhibition of this reflex in healthy volunteers, it facilitated the reflex in IBS[139]. Proof of altered brain activity has been shown with brain imaging studies and the potential of this research should be further explored[140]. The current know-how on brain imaging can be extensively consulted in review articles by Van Oudenhove *et al*[141], Smith *et al*[142] and Mayer *et al*[57,140].

Sensitized ascending and descending pathways: Upon repetitive stimulation by extrinsic primary afferent neurons, intracellular signaling cascades are activated within the spinal dorsal horn neurons. This leads to amplified responses to both innocuous and noxious input due to two major mechanisms: the facilitation of excitatory synaptic responses (so-called wind-up) and the downregulation of descending inhibitory influences[47,143]. The main mediator of wind-up is the neurotransmitter glutamate. When the presynaptic release of glutamate is triggered, glutamate acts on the ligand-gated ion channels NMDA (*N*-methyl-D-aspartate) receptors, kainate, AMPA (*a*-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid) and metabotropic glutamate receptors (mGLUR) expressed by the dorsal horn neurons. In addition to this direct effect, hyperstimulation of spinal neurons phosphorylates NMDA receptors which further increases NMDA receptor responsiveness to glutamate and increases synaptic strength[144]. AMPA receptor trafficking from the intracellular stores to the synaptic plasma membrane has also shown to augment glutamate responsiveness in a mice model of visceral nociception induced by intracolonic capsaicin[145]. The potential therapeutic effect of glutamate removal has also been investigated in experimental animal models. In this regard, it has been shown that ceftriaxone attenuates visceral hypersensitivity to CRD in rats with DSS and TNBS colitis. This effect was mediated *via* overexpression of spinal glutamate transporter-1 which increased removal of extracellular glutamate[146]. Other important mediators of central sensitization include substance P (SP), PGE_2 and brain-derived neurotropic factor which respectively target spinal neurokinin-1 receptor expression, PGE_2 receptors and tyrosine kinase B receptors[147]. For example, PGE_2 suppresses glycinergic transmission *via* activation PGE_2 receptors of the EP_2 subtype and subsequent PKA-dependent blockade of glycine receptors containing the $\alpha 3$ subunit ($GlyR\alpha 3$)[148]. The result of this blockade is the discontinuance of dorsal horn nociceptive neurons from their inhibitory control by glycinergic neurons. This PGE_2-evoked mechanism facilitates nociceptive input from the spinal cord. Similarly, a loss of GABAergic synaptic inhibition also increases nociceptive signaling[149]. COX-2, the enzyme that forms PGE_2 is markedly upregulated in the spinal cord during acute and chronic peripheral inflammation. In the spinal cord, basal release of PGE_2 is increased after peripheral inflammation[150]. Apart from neuron-neuron interactions, also glial cell-nerve interactions modulate signaling at the neuronal synapse, although this research is still in its infancy. Spinal glial cell activation is believed to be important in facilitation of nociceptive signals in various pain conditions. Under physiological conditions, glial cells are quiescent. However, during inflammation glial cells produce a variety of nociceptive agents such as TNFα, IL-1 and NO[151]. Most information has been obtained from experimental animal models of injury[152]. For instance, it has been shown that neonatal colonic irritation-induced visceral hypersensitivity in rats is accompanied by an increased expression of OX42, indicating glial cell proliferation. Visceral hypersensitivity was blocked with minocycline, an inhibitor of glial cell activation[153]. Recently, morphological remodeling of colonic afferent central nerve terminals was proposed in a mice model of hypersensitivity after TNBS inflammation. However, overall the "sprouting" theory of central afferent colonic nerve endings as a mechanism of central sensitization remains controversial[154]. Studies using functional brain imaging techniques have shown inflammation-induced modulation of activity in brain regions involved in visceral sensation, such as the ACC of the limbic system.

Electrophysiological studies in laboratory animals have shown that ACC sensitization occurs in viscerally hypersensitive rats[155]. It was revealed that for instance IBS was associated with decreased gray matter density in various brain areas, including medial and ventrolateral prefrontal cortex, posterior parietal cortex, ventral striatum, thalamus, and PAG. Further, IBS patients show brain responses consistent with hyperresponsiveness to gut distension in terms of vigilance, arousal and perhaps sensory sensitization[156]. Taken together, emerging evidence of structural brain changes in IBS is intriguing, but should be interpreted with great caution until more knowledge about the nature and implications of the observed alterations becomes available[63,157].

Accumulating evidence also suggests that descending facilitatory influences may contribute to the development and maintenance of hyperalgesia and thus contribute to chronic pain states. In this regard, a role for the RVM in the maintenance of hyperalgesic states following peripheral tissue injury activated by NMDA receptors, neurotensin receptors and NO is established[58]. Impaired ability to activate the descending pain inhibitory system has been hypothesized in IBS[57]. Aside from IBS patients, patients with active UC have been reported with reduced threshold to perception and reduced maximal tolerance to anorectal balloon distension[158]. CD children and adolescents suffering from abdominal pain despite remission had a lower rectal sensory pain threshold compared to healthy patients in a study conducted by Faure and coworkers[159]. Paradoxically, in other studies conducted in

chronic quiescent intestinal inflammatory states such as CD or UC, patients experience attenuated rectal perception and increased threshold for discomfort. UC patients with mild mucosal inflammation of the rectum had lower thresholds for discomfort during rectosigmoid distension compared to healthy patients[2]. A central descending inhibitory mechanism of sensory pathways in chronic inflammatory states, which would not be active in IBS patients, might be responsible for this seemingly discrepancy. This concept is further supported by a study showing that colonic inflammation is not necessarily associated with increased afferent input to the brain and that, in response to colorectal distension, inhibition of limbic/paralimbic circuits was observed in UC and control patients, but not in IBS patients[57]. Strong inhibitory mechanisms counteracting inflammation-induced hypersensitivity can be activated in chronic inflammatory pathologies, but seem to be deficient in patients with IBS-associated visceral hypersensitivity[160].

A recent meta-analysis of published studies on brain responses to rectal distension have shown differences between IBS patients and healthy controls[161]. Recently, Larsson and co-workers have shown that hypersensitive patients with IBS had greater activation of the insula and reduced deactivation in the pregenual ACC during noxious rectal distension compared to healthy patients and normosensitive IBS patients[162].

FUTURE DIRECTIONS

New therapeutic strategies may arise from the progressing identification of molecular prognostic markers and characterization of the molecular basis of IBD and IBS. Recently, dysfunction of microRNAs (miRNAs), which are non-coding RNA molecules that regulate gene expression, was postulated to play a role in IBD and IBS. Wu *et al*[163] showed that miRNAs regulate colonic epithelial cell-derived chemokine expression and that colonic tissues from patients with ulcerative colitis have altered miRNA expression patterns. An increased expression of miR-29a was also observed in blood microvesicles, small bowel and colon tissues of IBS patients with increased intestinal membrane permeability[164]. Recent evidence suggests that miR-29 expression is upregulated in human dendritic cells in response to NOD2 signals with concomitant down-regulation of interleukin-23[165]. Interestingly, dendritic cells with NOD2 polymorphisms from Crohn's disease patients fail to induce miR-29 upon pattern recognition receptor stimulation[165]. Moreover, experimental colitis in miR-29-deficient mice is more severe and associated with significantly enhanced levels of IL-23 and T helper 17 in the intestinal mucosa[165]. In respect to visceral pain, it was recently suggested that epigenetic central mechanisms are involved in the regulation of stress-induced visceral hypersensitivity in rats[166]. Overall, these results suggest that modulation of genetic and epigenetic regulatory mechanisms and profiling of miRNAs may represent promising strategies for the treatment of pain associated with IBD and IBS.

CONCLUSION

Chronic abdominal pain in IBD and IBS requires notion of how the lower gut becomes highly sensitive to any kind of stimulus. Noninvasive markers, including PET and fMRI, combined with pharmacology are used to assess hypersensitivity in these pathologies. In support, functional anatomical and physiological studies in rodents are being conducted[167]. Together these approaches discovered a significant amount of the neuroanatomical substrates and molecules in gut hypersensitivity, yet the degree to which each of these mechanisms contribute to hypersensitivity remains unknown. In both IBD and IBS, the complex interplay of sensitization occurs at different sites of action among the brain-gut axis and can be broadly categorized: sensitization of visceral afferents, sensitization of spinal cord ascending afferents, altered descending excitatory and inhibitory influences to the spinal cord nociceptive neurons and misinterpretation of non-noxious sensation as noxious due to cognitive and emotional biasing (hypervigilance)[47].

IBS and IBD have many of the mechanisms and molecules in visceral peripheral and CNS sensitization in common. Currently, no unambiguous neuronal marker exists that discriminates IBD and IBS. However, an inactive descending inhibitory control is hypothesized in IBS, but not in IBD. A recent study has suggested differences in coping behavior between IBD and IBS[168]. These differences among IBD and IBS certainly merit more appraisal, however, should be interpreted with caution. There are proportionally less studies on sensitivity in IBD patients than in IBS patients, therefore making head-to-head comparisons difficult. The shortage of studies on sensitivity in IBD patients may be attributed to the risk of jeopardizing remission by the barostat-induced distensions performed for sensitivity measurements. Nevertheless in both cases neuroplastic changes are quite common and the observed differences may not per se reflect disorder-specific changes, but may be attributed to affective disturbances, negative emotions in anticipation of/during visceral stimulation, and altered pain-related expectations and learning processes[63]. Expectation of pain may explain up to 50% of the variation of pain ratings[169]. Indeed, abdominal pain is not linearly related to peripheral sensory input. A considerable proportion (about one third) of IBS patients have a normal rectal perception, and a proportion of both UC and CD patients had increased thresholds for perception and discomfort[2]. Due to the multi-factorial complexity of sensitization in IBD and IBS, there is presently a rather limited success of available therapeutic approaches for IBS and the functional IBS-like symptoms in IBD. Technological progress that allows mapping of sensitization may be interesting to screen patients. In patients with peripheral sensitization mechanisms, the combination of anti-inflammatory properties and analgesic properties within one drug

seems a promising route for translational research. In patients with CNS disorders, approaches such as cognitive therapy or anti-depressive agents that decrease anxiety or hypervigilance might be beneficial.

In summary, the mechanisms of hypersensitivity in response to bowel inflammation are complex and need to be further unraveled. Determining the level of sensitization is crucial for the assessment of disease activity and for tailoring therapy. Several of these already defined mechanisms can be coined up as potential targets for the development of therapeutic options for inflammatory GI pain.

REFERENCES

1 **Wagtmans MJ**, Verspaget HW, Lamers CB, van Hogezand RA. Crohn's disease in the elderly: a comparison with young adults. *J Clin Gastroenterol* 1998; **27**: 129-133 [PMID: 9754773 DOI: 10.1097/00004836-199809000-00005]

2 **Chang L**, Munakata J, Mayer EA, Schmulson MJ, Johnson TD, Bernstein CN, Saba L, Naliboff B, Anton PA, Matin K. Perceptual responses in patients with inflammatory and functional bowel disease. *Gut* 2000; **47**: 497-505 [PMID: 10986209 DOI: 10.1136/gut.47.4.497]

3 **Geboes K**, Collins S. Structural abnormalities of the nervous system in Crohn's disease and ulcerative colitis. *Neurogastroenterol Motil* 1998; **10**: 189-202 [PMID: 9659662 DOI: 10.1046/j.1365-2982.1998.00102.x]

4 **De Schepper HU**, De Man JG, Moreels TG, Pelckmans PA, De Winter BY. Review article: gastrointestinal sensory and motor disturbances in inflammatory bowel disease - clinical relevance and pathophysiological mechanisms. *Aliment Pharmacol Ther* 2008; **27**: 621-637 [PMID: 18221407 DOI: 10.1111/j.1365-2036.2008.03624.x]

5 **Cross RK**, Wilson KT, Binion DG. Narcotic use in patients with Crohn's disease. *Am J Gastroenterol* 2005; **100**: 2225-2229 [PMID: 16181373 DOI: 10.1111/j.1572-0241.2005.00256.x]

6 **Farrokhyar F**, Marshall JK, Easterbrook B, Irvine EJ. Functional gastrointestinal disorders and mood disorders in patients with inactive inflammatory bowel disease: prevalence and impact on health. *Inflamm Bowel Dis* 2006; **12**: 38-46 [PMID: 16374257 DOI: 10.1097/01.MIB.0000195391.49762.89]

7 **Camilleri M**. Management of the irritable bowel syndrome. *Gastroenterology* 2001; **120**: 652-668 [PMID: 11179242 DOI: 10.1053/gast.2001.21908]

8 **Tibble JA**, Sigthorsson G, Foster R, Forgacs I, Bjarnason I. Use of surrogate markers of inflammation and Rome criteria to distinguish organic from nonorganic intestinal disease. *Gastroenterology* 2002; **123**: 450-460 [PMID: 12145798 DOI: 10.1053/gast.2002.34755]

9 **Grad C**, David L, Portincasa P, Dumitraşcu DL. Diagnostic value of calprotectin in irritable bowel syndrome and in inflammatory bowel disease. *Rom J Intern Med* 2012; **50**: 3-6 [PMID: 22788087]

10 **Dinan TG**, Quigley EM, Ahmed SM, Scully P, O'Brien S, O'Mahony L, O'Mahony S, Shanahan F, Keeling PW. Hypothalamic-pituitary-gut axis dysregulation in irritable bowel syndrome: plasma cytokines as a potential biomarker? *Gastroenterology* 2006; **130**: 304-311 [PMID: 16472586 DOI: 10.1053/j.gastro.2005.11.033]

11 **Chadwick VS**, Chen W, Shu D, Paulus B, Bethwaite P, Tie A, Wilson I. Activation of the mucosal immune system in irritable bowel syndrome. *Gastroenterology* 2002; **122**: 1778-1783 [PMID: 12055584 DOI: 10.1053/gast.2002.33579]

12 **Spiller RC**. Potential biomarkers. *Gastroenterol Clin North Am* 2011; **40**: 121-139 [PMID: 21333904 DOI: 10.1016/j.gtc.2011.01.001]

13 **Swan C**, Duroudier NP, Campbell E, Zaitoun A, Hastings M, Dukes GE, Cox J, Kelly FM, Wilde J, Lennon MG, Neal KR, Whorwell PJ, Hall IP, Spiller RC. Identifying and testing candidate genetic polymorphisms in the irritable bowel syndrome (IBS): association with TNFSF15 and TNFα. *Gut* 2013; **62**: 985-994 [PMID: 22684480 DOI: 10.1136/gutjnl-2011-301213]

14 **Spiller R**, Garsed K. Postinfectious irritable bowel syndrome. *Gastroenterology* 2009; **136**: 1979-1988 [PMID: 19457422 DOI: 10.1053/j.gastro.2009.02.074]

15 **Podolsky DK**. Inflammatory bowel disease. *N Engl J Med* 2002; **347**: 417-429 [PMID: 12167685 DOI: 10.1056/NEJMra020831]

16 **Malykhina AP**. Neural mechanisms of pelvic organ cross-sensitization. *Neuroscience* 2007; **149**: 660-672 [PMID: 17920206 DOI: 10.1016/j.neuroscience.2007.07.053]

17 **Timmermans JP**, Scheuermann DW, Stach W, Adriaensen D, De Groodt-Lasseel MH. Functional morphology of the enteric nervous system with special reference to large mammals. *Eur J Morphol* 1992; **30**: 113-122 [PMID: 1457247]

18 **Costa M**, Brookes SJ, Hennig GW. Anatomy and physiology of the enteric nervous system. *Gut* 2000; **47** Suppl 4: iv15-iv19; discussion iv26 [PMID: 11076898]

19 **Furness JB**. The organisation of the autonomic nervous system: peripheral connections. *Auton Neurosci* 2006; **130**: 1-5 [PMID: 16798102 DOI: 10.1016/j.autneu.2006.05.003]

20 **Chey WY**, Jin HO, Lee MH, Sun SW, Lee KY. Colonic motility abnormality in patients with irritable bowel syndrome exhibiting abdominal pain and diarrhea. *Am J Gastroenterol* 2001; **96**: 1499-1506 [PMID: 11374689 DOI: 10.1111/j.1572-0241.2001.03804.x]

21 **Ford AC**, Talley NJ, Spiegel BM, Foxx-Orenstein AE, Schiller L, Quigley EM, Moayyedi P. Effect of fibre, antispasmodics, and peppermint oil in the treatment of irritable bowel syndrome: systematic review and meta-analysis. *BMJ* 2008; **337**: a2313 [PMID: 19008265 DOI: 10.1136/bmj.a2313]

22 **Makharia GK**. Understanding and treating abdominal pain and spasms in organic gastrointestinal diseases: inflammatory bowel disease and biliary diseases. *J Clin Gastroenterol* 2011; **45** Suppl: S89-S93 [PMID: 21666426 DOI: 10.1097/MCG.0b013e31821fbd82]

23 **Blackshaw LA**, Brookes SJ, Grundy D, Schemann M. Sensory transmission in the gastrointestinal tract. *Neurogastroenterol Motil* 2007; **19**: 1-19 [PMID: 17280582 DOI: 10.1111/j.1365-2982.2006.00871.x]

24 **Powley TL**, Wang XY, Fox EA, Phillips RJ, Liu LW, Huizinga JD. Ultrastructural evidence for communication between intramuscular vagal mechanoreceptors and interstitial cells of Cajal in the rat fundus. *Neurogastroenterol Motil* 2008; **20**: 69-79 [PMID: 17931338]

25 **Grundy D**. Neuroanatomy of visceral nociception: vagal and splanchnic afferent. *Gut* 2002; **51** Suppl 1: i2-i5 [PMID: 12077054 DOI: 10.1136/gut.51.suppl_1.i2]

26 **Page AJ**, Martin CM, Blackshaw LA. Vagal mechanoreceptors and chemoreceptors in mouse stomach and esophagus. *J Neurophysiol* 2002; **87**: 2095-2103 [PMID: 11929927]

27 **Holzer P**. Afferent signalling of gastric acid challenge. *J Physiol Pharmacol* 2003; **54** Suppl 4: 43-53 [PMID: 15075448]

28 **Gschossmann JM**, Mayer EA, Miller JC, Raybould HE. Subdiaphragmatic vagal afferent innervation in activation of an opioidergic antinociceptive system in response to colorectal distension in rats. *Neurogastroenterol Motil* 2002; **14**: 403-408 [PMID: 12213108 DOI: 10.1046/j.1365-2982.2002.00345.x]

29 **Chen SL**, Wu XY, Cao ZJ, Fan J, Wang M, Owyang C, Li Y. Subdiaphragmatic vagal afferent nerves modulate visceral pain. *Am J Physiol Gastrointest Liver Physiol* 2008; **294**: G1441-G1449 [PMID: 18420825 DOI: 10.1152/ajpgi.00588.2007]

30 **Holtmann G**, Goebell H, Jockenhoevel F, Talley NJ. Altered vagal and intestinal mechanosensory function in chronic un-

explained dyspepsia. *Gut* 1998; **42**: 501-506 [PMID: 9616311 DOI: 10.1136/gut.42.4.501]
31 **Randich A**, Gebhart GF. Vagal afferent modulation of nociception. *Brain Res Brain Res Rev* 1992; **17**: 77-99 [PMID: 1327371 DOI: 10.1016/0165-017392)90009-B]
32 **Matteoli G**, Boeckxstaens GE. The vagal innervation of the gut and immune homeostasis. *Gut* 2013; **62**: 1214-1222 [PMID: 23023166 DOI: 10.1136/gutjnl-2012-302550]
33 **Christianson JA**, Traub RJ, Davis BM. Differences in spinal distribution and neurochemical phenotype of colonic afferents in mouse and rat. *J Comp Neurol* 2006; **494**: 246-259 [PMID: 16320237 DOI: 10.1002/cne.20816]
34 **Brierley SM**, Jones RC, Gebhart GF, Blackshaw LA. Splanchnic and pelvic mechanosensory afferents signal different qualities of colonic stimuli in mice. *Gastroenterology* 2004; **127**: 166-178 [PMID: 15236183 DOI: 10.1053/j.gastro.2004.04.008]
35 **Gué M**, Junien JL, Buéno L. The kappa agonist fedotozine modulates colonic distention-induced inhibition of gastric motility and emptying in dogs. *Gastroenterology* 1994; **107**: 1327-1334 [PMID: 7926497 DOI: 10.1016/0016-508594)90534-7]
36 **De Schepper HU**, De Man JG, Van Nassauw L, Timmermans JP, Herman AG, Pelckmans PA, De Winter BY. Acute distal colitis impairs gastric emptying in rats via an extrinsic neuronal reflex pathway involving the pelvic nerve. *Gut* 2007; **56**: 195-202 [PMID: 16973715 DOI: 10.1136/gut.2006.104745]
37 **Traub RJ**. Evidence for thoracolumbar spinal cord processing of inflammatory, but not acute colonic pain. *Neuroreport* 2000; **11**: 2113-2116 [PMID: 10923654 DOI: 10.1097/00001756-200007140-00011]
38 **Grundy D**. Speculations on the structure/function relationship for vagal and splanchnic afferent endings supplying the gastrointestinal tract. *J Auton Nerv Syst* 1988; **22**: 175-180 [PMID: 3047201 DOI: 10.1016/0165-1838(88)90104-X]
39 **Lamb K**, Kang YM, Gebhart GF, Bielefeldt K. Gastric inflammation triggers hypersensitivity to acid in awake rats. *Gastroenterology* 2003; **125**: 1410-1418 [PMID: 14598257 DOI: 10.1016/j.gastro.2003.07.010]
40 **Lynn PA**, Blackshaw LA. In vitro recordings of afferent fibres with receptive fields in the serosa, muscle and mucosa of rat colon. *J Physiol* 1999; **518** (Pt 1): 271-282 [PMID: 10373708 DOI: 10.1111/j.1469-7793.1999.0271r.x]
41 **Song X**, Chen BN, Zagorodnyuk VP, Lynn PA, Blackshaw LA, Grundy D, Brunsden AM, Costa M, Brookes SJ. Identification of medium/high-threshold extrinsic mechanosensitive afferent nerves to the gastrointestinal tract. *Gastroenterology* 2009; **137**: 274-284, 284.e1 [PMID: 19268671]
42 **Traub RJ**, Murphy A. Colonic inflammation induces fos expression in the thoracolumbar spinal cord increasing activity in the spinoparabrachial pathway. *Pain* 2002; **95**: 93-102 [PMID: 11790471 DOI: 10.1016/S0304-395901)00381-5]
43 **Ness TJ**, Metcalf AM, Gebhart GF. A psychophysiological study in humans using phasic colonic distension as a noxious visceral stimulus. *Pain* 1990; **43**: 377-386 [PMID: 2293146 DOI: 10.1016/0304-395990)90035-C]
44 **Urban MO**, Gebhart GF. Supraspinal contributions to hyperalgesia. *Proc Natl Acad Sci USA* 1999; **96**: 7687-7692 [PMID: 10393881 DOI: 10.1073/pnas.96.14.7687]
45 **Gybels JM**, Sweet WH. Neurosurgical treatment of persistent pain. Physiological and pathological mechanisms of human pain. *Pain Headache* 1989; **11**: 1-402 [PMID: 2666976]
46 **Pasricha PJ**, Willis WD, Gebhart GF. Chronic Abdominal and Visceral Pain: Theory and Practice. 1 st ed. New York: Informa, 2006
47 **Anand P**, Aziz Q, Willert R, van Oudenhove L. Peripheral and central mechanisms of visceral sensitization in man. *Neurogastroenterol Motil* 2007; **19**: 29-46 [PMID: 17280584 DOI: 10.1111/j.1365-2982.2006.00873.x]
48 **Wouters MM**, Van Wanrooy S, Casteels C, Nemethova A, de Vries A, Van Oudenhove L, Van den Wijngaard RM, Van Laere K, Boeckxstaens G. Altered brain activation to colorectal distention in visceral hypersensitive maternal-separated rats. *Neurogastroenterol Motil* 2012; **24**: 678-685, e297 [PMID: 22509925 DOI: 10.1111/j.1365-2982.2012.01919.x.]
49 **Rosenberger C**, Thürling M, Forsting M, Elsenbruch S, Timmann D, Gizewski ER. Contributions of the cerebellum to disturbed central processing of visceral stimuli in irritable bowel syndrome. *Cerebellum* 2013; **12**: 194-198 [PMID: 22910984 DOI: 10.1007/s12311-012-0413-3]
50 **Al-Chaer ED**, Feng Y, Willis WD. A role for the dorsal column in nociceptive visceral input into the thalamus of primates. *J Neurophysiol* 1998; **79**: 3143-3150 [PMID: 9636114]
51 **Ness TJ**. Evidence for ascending visceral nociceptive information in the dorsal midline and lateral spinal cord. *Pain* 2000; **87**: 83-88 [PMID: 10863048 DOI: 10.1016/S0304-395900)00272-4]
52 **Palecek J**, Paleckova V, Willis WD. The roles of pathways in the spinal cord lateral and dorsal funiculi in signaling nociceptive somatic and visceral stimuli in rats. *Pain* 2002; **96**: 297-307 [PMID: 11973002 DOI: 10.1016/S0304-395901)00459-6]
53 **Palecek J**. The role of dorsal columns pathway in visceral pain. *Physiol Res* 2004; **53** Suppl 1: S125-S130 [PMID: 15119943]
54 **Danziger N**, Rémy P, Pidoux B, Dormont D, Samson Y, Fournier E, Wall PD, Willer JC. A clinical and neurophysiological study of a patient with an extensive transection of the spinal cord sparing only a part of one anterolateral quadrant. *Brain* 1996; **119** (Pt 6): 1835-1848 [PMID: 9009991 DOI: 10.1093/brain/119.6.1835]
55 **Reynolds DV**. Surgery in the rat during electrical analgesia induced by focal brain stimulation. *Science* 1969; **164**: 444-445 [PMID: 4887743 DOI: 10.1126/science.164.3878.444]
56 **Gebhart GF**. Descending modulation of pain. *Neurosci Biobehav Rev* 2004; **27**: 729-737 [PMID: 15019423 DOI: 10.1016/j.neubiorev.2003.11.008]
57 **Mayer EA**, Berman S, Suyenobu B, Labus J, Mandelkern MA, Naliboff BD, Chang L. Differences in brain responses to visceral pain between patients with irritable bowel syndrome and ulcerative colitis. *Pain* 2005; **115**: 398-409 [PMID: 15911167 DOI: 10.1016/j.pain.2005.03.023]
58 **Brookes SJ**, Spencer NJ, Costa M, Zagorodnyuk VP. Extrinsic primary afferent signalling in the gut. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 286-296 [PMID: 23438947 DOI: 10.1038/nrgastro.2013.29]
59 **Farmer AD**, Aziz Q. Visceral pain hypersensitivity in functional gastrointestinal disorders. *Br Med Bull* 2009; **91**: 123-136 [PMID: 19620136 DOI: 10.1093/bmb/ldp026]
60 **Straub RH**, Wiest R, Strauch UG, Härle P, Schölmerich J. The role of the sympathetic nervous system in intestinal inflammation. *Gut* 2006; **55**: 1640-1649 [PMID: 17047110 DOI: 10.1136/gut.2006.091322]
61 **Merskey H**, Bogduk N. Classification of Chronic Pain. In: Harold Merskey, Nikolai Bogduk. Part III: Pain Terms, A Current List with Definitions and Notes on Usage. Seattle: IASP Press, 1994: 209-214
62 **Mertz HR**. Irritable bowel syndrome. *N Engl J Med* 2003; **349**: 2136-2146 [PMID: 14645642 DOI: 10.1056/NEJMra035579]
63 **Elsenbruch S**. Abdominal pain in Irritable Bowel Syndrome: a review of putative psychological, neural and neuroimmune mechanisms. *Brain Behav Immun* 2011; **25**: 386-394 [PMID: 21094682 DOI: 10.1016/j.bbi.2010.11.010]
64 **Posserud I**, Syrous A, Lindström L, Tack J, Abrahamsson H, Simrén M. Altered rectal perception in irritable bowel syndrome is associated with symptom severity. *Gastroenterology* 2007; **133**: 1113-1123 [PMID: 17919487 DOI: 10.1053/j.gastro.2007.07.024]
65 **Bouin M**, Plourde V, Boivin M, Riberdy M, Lupien F, Laganière M, Verrier P, Poitras P. Rectal distention testing in patients with irritable bowel syndrome: sensitivity, specificity, and predictive values of pain sensory thresholds. *Gastroen-*

terology 2002; **122**: 1771-1777 [PMID: 12055583 DOI: 10.1053/gast.2002.33601]
66 **Hazlett-Stevens H**, Craske MG, Mayer EA, Chang L, Naliboff BD. Prevalence of irritable bowel syndrome among university students: the roles of worry, neuroticism, anxiety sensitivity and visceral anxiety. *J Psychosom Res* 2003; **55**: 501-505 [PMID: 14642979 DOI: 10.1016/S0022-399903)00019-9]
67 **De Winter BY**, De Man JG. Interplay between inflammation, immune system and neuronal pathways: effect on gastrointestinal motility. *World J Gastroenterol* 2010; **16**: 5523-5535 [PMID: 21105185 DOI: 10.3748/wjg.v16.i44.5523]
68 **Bueno L**, de Ponti F, Fried M, Kullak-Ublick GA, Kwiatek MA, Pohl D, Quigley EM, Tack J, Talley NJ. Serotonergic and non-serotonergic targets in the pharmacotherapy of visceral hypersensitivity. *Neurogastroenterol Motil* 2007; **19**: 89-119 [PMID: 17280587 DOI: 10.1111/j.1365-2982.2006.00876.x]
69 **Dunlop SP**, Jenkins D, Neal KR, Naesdal J, Borgaonker M, Collins SM, Spiller RC. Randomized, double-blind, placebo-controlled trial of prednisolone in post-infectious irritable bowel syndrome. *Aliment Pharmacol Ther* 2003; **18**: 77-84 [PMID: 12848628 DOI: 10.1046/j.1365-2036.2003.01640.x]
70 **Wang H**, Steeds J, Motomura Y, Deng Y, Verma-Gandhu M, El-Sharkawy RT, McLaughlin JT, Grencis RK, Khan WI. CD4+ T cell-mediated immunological control of enterochromaffin cell hyperplasia and 5-hydroxytryptamine production in enteric infection. *Gut* 2007; **56**: 949-957 [PMID: 17303597 DOI: 10.1136/gut.2006.103226]
71 **Gershon MD**. Nerves, reflexes, and the enteric nervous system: pathogenesis of the irritable bowel syndrome. *J Clin Gastroenterol* 2005; **39**: S184-S193 [PMID: 15798484 DOI: 10.1097/01.mcg.0000156403.37240.30]
72 **Greenwood-Van Meerveld B**, Venkova K, Hicks G, Dennis E, Crowell MD. Activation of peripheral 5-HT receptors attenuates colonic sensitivity to intraluminal distension. *Neurogastroenterol Motil* 2006; **18**: 76-86 [PMID: 16371086 DOI: 10.1111/j.1365-2982.2005.00723.x]
73 **Coffin B**, Farmachidi JP, Rueegg P, Bastie A, Bouhassira D. Tegaserod, a 5-HT4 receptor partial agonist, decreases sensitivity to rectal distension in healthy subjects. *Aliment Pharmacol Ther* 2003; **17**: 577-585 [PMID: 12622767 DOI: 10.1046/j.1365-2036.2003.01449.x]
74 **Spiller RC**, Jenkins D, Thornley JP, Hebden JM, Wright T, Skinner M, Neal KR. Increased rectal mucosal enteroendocrine cells, T lymphocytes, and increased gut permeability following acute Campylobacter enteritis and in post-dysenteric irritable bowel syndrome. *Gut* 2000; **47**: 804-811 [PMID: 11076879 DOI: 10.1136/gut.47.6.804]
75 **Dunlop SP**, Jenkins D, Neal KR, Spiller RC. Relative importance of enterochromaffin cell hyperplasia, anxiety, and depression in postinfectious IBS. *Gastroenterology* 2003; **125**: 1651-1659 [PMID: 14724817 DOI: 10.1053/j.gastro.2003.09.028]
76 **Kim HS**, Lim JH, Park H, Lee SI. Increased immunoendocrine cells in intestinal mucosa of postinfectious irritable bowel syndrome patients 3 years after acute Shigella infection--an observation in a small case control study. *Yonsei Med J* 2010; **51**: 45-51 [PMID: 20046513 DOI: 10.3349/ymj.2010.51.1.45]
77 **Wang LH**, Fang XC, Pan GZ. Bacillary dysentery as a causative factor of irritable bowel syndrome and its pathogenesis. *Gut* 2004; **53**: 1096-1101 [PMID: 15247174 DOI: 10.1136/gut.2003.021154]
78 **Thabane M**, Marshall JK. Post-infectious irritable bowel syndrome. *World J Gastroenterol* 2009; **15**: 3591-3596 [PMID: 19653335 DOI: 10.3748/wjg.15.3591]
79 **Coates MD**, Mahoney CR, Linden DR, Sampson JE, Chen J, Blaszyk H, Crowell MD, Sharkey KA, Gershon MD, Mawe GM, Moses PL. Molecular defects in mucosal serotonin content and decreased serotonin reuptake transporter in ulcerative colitis and irritable bowel syndrome. *Gastroenterology* 2004; **126**: 1657-1664 [PMID: 15188158 DOI: 10.1053/j.gastro.2004.03.013]
80 **Gershon MD**. Review article: serotonin receptors and transporters -- roles in normal and abnormal gastrointestinal motility. *Aliment Pharmacol Ther* 2004; **20** Suppl 7: 3-14 [PMID: 15521849 DOI: 10.1111/j.1365-2036.2004.02180.x]
81 **Camilleri M**, Andrews CN, Bharucha AE, Carlson PJ, Ferber I, Stephens D, Smyrk TC, Urrutia R, Aerssens J, Thielemans L, Göhlmann H, van den Wyngaert I, Coulie B. Alterations in expression of p11 and SERT in mucosal biopsy specimens of patients with irritable bowel syndrome. *Gastroenterology* 2007; **132**: 17-25 [PMID: 17241856 DOI: 10.1053/j.gastro.2006.11.020]
82 **Coates MD**, Johnson AC, Greenwood-Van Meerveld B, Mawe GM. Effects of serotonin transporter inhibition on gastrointestinal motility and colonic sensitivity in the mouse. *Neurogastroenterol Motil* 2006; **18**: 464-471 [PMID: 16700726 DOI: 10.1111/j.1365-2982.2006.00792.x]
83 **Kumar S**, Ranjan P, Mittal B, Ghoshal UC. Serotonin transporter gene (SLC6A4) polymorphism in patients with irritable bowel syndrome and healthy controls. *J Gastrointestin Liver Dis* 2012; **21**: 31-38 [PMID: 22457857]
84 **Rijnierse A**, Nijkamp FP, Kraneveld AD. Mast cells and nerves tickle in the tummy: implications for inflammatory bowel disease and irritable bowel syndrome. *Pharmacol Ther* 2007; **116**: 207-235 [PMID: 17719089 DOI: 10.1016/j.pharmthera.2007.06.008]
85 **De Winter BY**, van den Wijngaard RM, de Jonge WJ. Intestinal mast cells in gut inflammation and motility disturbances. *Biochim Biophys Acta* 2012; **1822**: 66-73 [PMID: 21497195 DOI: 10.1016/j.bbadis.2011.03.016]
86 **Grundy D**. 5-HT system in the gut: roles in the regulation of visceral sensitivity and motor functions. *Eur Rev Med Pharmacol Sci* 2008; **12** Suppl 1: 63-67 [PMID: 18924445]
87 **Stead RH**, Dixon MF, Bramwell NH, Riddell RH, Bienenstock J. Mast cells are closely apposed to nerves in the human gastrointestinal mucosa. *Gastroenterology* 1989; **97**: 575-585 [PMID: 2666250]
88 **Van Nassauw L**, Adriaensen D, Timmermans JP. The bidirectional communication between neurons and mast cells within the gastrointestinal tract. *Auton Neurosci* 2007; **133**: 91-103 [PMID: 17169619 DOI: 10.1016/j.autneu.2006.10.003]
89 **Coldwell JR**, Phillis BD, Sutherland K, Howarth GS, Blackshaw LA. Increased responsiveness of rat colonic splanchnic afferents to 5-HT after inflammation and recovery. *J Physiol* 2007; **579**: 203-213 [PMID: 17138606 DOI: 10.1113/jphysiol.2006.123158]
90 **Ohashi K**, Sato Y, Kawai M, Kurebayashi Y. Abolishment of TNBS-induced visceral hypersensitivity in mast cell deficient rats. *Life Sci* 2008; **82**: 419-423 [PMID: 18222490 DOI: 10.1016/j.lfs.2007.11.027]
91 **Deiteren A**, De Man JG, Moreels TG, Pelckmans PA, De Winter BY. Mast cells are involved in post-inflammatory visceral hypersensitivity and exert their effects partially through H1 receptors. *Neurogastroenterol Motil* 2012; **24**: 152 (abstract)
92 **Klooker TK**, Braak B, Koopman KE, Welting O, Wouters MM, van der Heide S, Schemann M, Bischoff SC, van den Wijngaard RM, Boeckxstaens GE. The mast cell stabiliser ketotifen decreases visceral hypersensitivity and improves intestinal symptoms in patients with irritable bowel syndrome. *Gut* 2010; **59**: 1213-1221 [PMID: 20650926 DOI: 10.1136/gut.2010.213108]
93 **Raithel M**, Winterkamp S, Pacurar A, Ulrich P, Hochberger J, Hahn EG. Release of mast cell tryptase from human colorectal mucosa in inflammatory bowel disease. *Scand J Gastroenterol* 2001; **36**: 174-179 [PMID: 11252410 DOI: 10.1080/00365201750065933]
94 **Wang XY**, Zarate N, Soderholm JD, Bourgeois JM, Liu LW, Huizinga JD. Ultrastructural injury to interstitial cells of

Cajal and communication with mast cells in Crohn's disease. *Neurogastroenterol Motil* 2007; **19**: 349-364 [PMID: 17509017 DOI: 10.1111/j.1365-2982.2006.00894.x]
95 **Barbara G**, Stanghellini V, De Giorgio R, Corinaldesi R. Functional gastrointestinal disorders and mast cells: implications for therapy. *Neurogastroenterol Motil* 2006; **18**: 6-17 [PMID: 16371078]
96 **Fox CC**, Lazenby AJ, Moore WC, Yardley JH, Bayless TM, Lichtenstein LM. Enhancement of human intestinal mast cell mediator release in active ulcerative colitis. *Gastroenterology* 1990; **99**: 119-124 [PMID: 1693122]
97 **Sasaki Y**, Tanaka M, Kudo H. Differentiation between ulcerative colitis and Crohn's disease by a quantitative immunohistochemical evaluation of T lymphocytes, neutrophils, histiocytes and mast cells. *Pathol Int* 2002; **52**: 277-285 [PMID: 12031083 DOI: 10.1046/j.1440-1827.2002.01354.x]
98 **Cremon C**, Gargano L, Morselli-Labate AM, Santini D, Cogliandro RF, De Giorgio R, Stanghellini V, Corinaldesi R, Barbara G. Mucosal immune activation in irritable bowel syndrome: gender-dependence and association with digestive symptoms. *Am J Gastroenterol* 2009; **104**: 392-400 [PMID: 19174797 DOI: 10.1038/ajg.2008.94]
99 **Barbara G**, Stanghellini V, De Giorgio R, Cremon C, Cottrell GS, Santini D, Pasquinelli G, Morselli-Labate AM, Grady EF, Bunnett NW, Collins SM, Corinaldesi R. Activated mast cells in proximity to colonic nerves correlate with abdominal pain in irritable bowel syndrome. *Gastroenterology* 2004; **126**: 693-702 [PMID: 14988823 DOI: 10.1053/j.gastro.2003.11.055]
100 **Barbara G**, Wang B, Stanghellini V, de Giorgio R, Cremon C, Di Nardo G, Trevisani M, Campi B, Geppetti P, Tonini M, Bunnett NW, Grundy D, Corinaldesi R. Mast cell-dependent excitation of visceral-nociceptive sensory neurons in irritable bowel syndrome. *Gastroenterology* 2007; **132**: 26-37 [PMID: 17241857 DOI: 10.1053/j.gastro.2006.11.039]
101 **Hughes PA**, Brierley SM, Martin CM, Liebregts T, Persson J, Adam B, Holtmann G, Blackshaw LA. TRPV1-expressing sensory fibres and IBS: links with immune function. *Gut* 2009; **58**: 465-466 [PMID: 19211857 DOI: 10.1136/gut.2008.161760]
102 **Stenton GR**, Nohara O, Déry RE, Vliagoftis H, Gilchrist M, Johri A, Wallace JL, Hollenberg MD, Moqbel R, Befus AD. Proteinase-activated receptor (PAR)-1 and -2 agonists induce mediator release from mast cells by pathways distinct from PAR-1 and PAR-2. *J Pharmacol Exp Ther* 2002; **302**: 466-474 [PMID: 12130703 DOI: 10.1124/jpet.302.2.466]
103 **Vergnolle N**. Protease-activated receptors as drug targets in inflammation and pain. *Pharmacol Ther* 2009; **123**: 292-309 [PMID: 19481569 DOI: 10.1016/j.pharmthera.2009.05.004]
104 **Kim JA**, Choi SC, Yun KJ, Kim DK, Han MK, Seo GS, Yeom JJ, Kim TH, Nah YH, Lee YM. Expression of protease-activated receptor 2 in ulcerative colitis. *Inflamm Bowel Dis* 2003; **9**: 224-229 [PMID: 12902845 DOI: 10.1097/00054725-200307000-00002]
105 **Vergnolle N**. Clinical relevance of proteinase activated receptors (pars) in the gut. *Gut* 2005; **54**: 867-874 [PMID: 15888798 DOI: 10.1136/gut.2004.048876]
106 **Cenac N**, Coelho AM, Nguyen C, Compton S, Andrade-Gordon P, MacNaughton WK, Wallace JL, Hollenberg MD, Bunnett NW, Garcia-Villar R, Bueno L, Vergnolle N. Induction of intestinal inflammation in mouse by activation of proteinase-activated receptor-2. *Am J Pathol* 2002; **161**: 1903-1915 [PMID: 12414536 DOI: 10.1016/S0002-944010)64466-5]
107 **Cenac N**, Andrews CN, Holzhausen M, Chapman K, Cottrell G, Andrade-Gordon P, Steinhoff M, Barbara G, Beck P, Bunnett NW, Sharkey KA, Ferraz JG, Shaffer E, Vergnolle N. Role for protease activity in visceral pain in irritable bowel syndrome. *J Clin Invest* 2007; **117**: 636-647 [PMID: 17304351 DOI: 10.1172/JCI29255]
108 **Bradesi S**, Herman J, Mayer EA. Visceral analgesics: drugs with a great potential in functional disorders? *Curr Opin Pharmacol* 2008; **8**: 697-703 [PMID: 18786654 DOI: 10.1016/j.coph.2008.08.009]
109 **Grant AD**, Cottrell GS, Amadesi S, Trevisani M, Nicoletti P, Materazzi S, Altier C, Cenac N, Zamponi GW, Bautista-Cruz F, Lopez CB, Joseph EK, Levine JD, Liedtke W, Vanner S, Vergnolle N, Geppetti P, Bunnett NW. Protease-activated receptor 2 sensitizes the transient receptor potential vanilloid 4 ion channel to cause mechanical hyperalgesia in mice. *J Physiol* 2007; **578**: 715-733 [PMID: 17124270 DOI: 10.1113/jphysiol.2006.121111]
110 **Cenac N**, Altier C, Motta JP, d'Aldebert E, Galeano S, Zamponi GW, Vergnolle N. Potentiation of TRPV4 signalling by histamine and serotonin: an important mechanism for visceral hypersensitivity. *Gut* 2010; **59**: 481-488 [PMID: 20332520 DOI: 10.1136/gut.2009.192567]
111 **Cattaruzza F**, Spreadbury I, Miranda-Morales M, Grady EF, Vanner S, Bunnett NW. Transient receptor potential ankyrin-1 has a major role in mediating visceral pain in mice. *Am J Physiol Gastrointest Liver Physiol* 2010; **298**: G81-G91 [PMID: 19875705 DOI: 10.1152/ajpgi.00221.2009]
112 **Sharkey KA**, Mawe GM. Neuroimmune and epithelial interactions in intestinal inflammation. *Curr Opin Pharmacol* 2002; **2**: 669-677 [PMID: 12482729 DOI: 10.1016/S1471-489202)00215-1]
113 **Delafoy L**, Raymond F, Doherty AM, Eschalier A, Diop L. Role of nerve growth factor in the trinitrobenzene sulfonic acid-induced colonic hypersensitivity. *Pain* 2003; **105**: 489-497 [PMID: 14527709 DOI: 10.1016/S0304-395903)00266-5]
114 **di Mola FF**, Friess H, Zhu ZW, Koliopanos A, Bley T, Di Sebastiano P, Innocenti P, Zimmermann A, Büchler MW. Nerve growth factor and Trk high affinity receptor (TrkA) gene expression in inflammatory bowel disease. *Gut* 2000; **46**: 670-679 [PMID: 10764711 DOI: 10.1136/gut.46.5.670]
115 **Rühl A**. Glial cells in the gut. *Neurogastroenterol Motil* 2005; **17**: 777-790 [PMID: 16336493 DOI: 10.1111/j.1365-2982.2005.00687.x]
116 **Vanden Berghe P**, Tack J, Boesmans W. Highlighting synaptic communication in the enteric nervous system. *Gastroenterology* 2008; **135**: 20-23 [PMID: 18555020 DOI: 10.1053/j.gastro.2008.06.001]
117 **De Filippis D**, Esposito G, Cirillo C, Cipriano M, De Winter BY, Scuderi C, Sarnelli G, Cuomo R, Steardo L, De Man JG, Iuvone T. Cannabidiol reduces intestinal inflammation through the control of neuroimmune axis. *PLoS One* 2011; **6**: e28159 [PMID: 22163000 DOI: 10.1371/journal.pone.0028159]
118 **Neunlist M**, Van Landeghem L, Bourreille A, Savidge T. Neuro-glial crosstalk in inflammatory bowel disease. *J Intern Med* 2008; **263**: 577-583 [PMID: 18479256 DOI: 10.1111/j.1365-2796.2008.01963.x]
119 **Cirillo C**, Sarnelli G, Esposito G, Grosso M, Petruzzelli R, Izzo P, Calì G, D'Armiento FP, Rocco A, Nardone G, Iuvone T, Steardo L, Cuomo R. Increased mucosal nitric oxide production in ulcerative colitis is mediated in part by the enteroglial-derived S100B protein. *Neurogastroenterol Motil* 2009; **21**: 1209-e112 [PMID: 19558426 DOI: 10.1111/j.1365-2982.2009.01346.x]
120 **Roberts PJ**, Morgan K, Miller R, Hunter JO, Middleton SJ. Neuronal COX-2 expression in human myenteric plexus in active inflammatory bowel disease. *Gut* 2001; **48**: 468-472 [PMID: 11247889 DOI: 10.1136/gut.48.4.468]
121 **Mollace V**, Muscoli C, Masini E, Cuzzocrea S, Salvemini D. Modulation of prostaglandin biosynthesis by nitric oxide and nitric oxide donors. *Pharmacol Rev* 2005; **57**: 217-252 [PMID: 15914468 DOI: 10.1124/pr.57.2.1]
122 **Holzer P**. Transient receptor potential (TRP) channels as drug targets for diseases of the digestive system. *Pharmacol Ther* 2011; **131**: 142-170 [PMID: 21420431 DOI: 10.1016/j.pharmthera.2011.03.006]
123 **Brierley SM**, Jones RC, Xu L, Gebhart GF, Blackshaw LA. Activation of splanchnic and pelvic colonic afferents by bradykinin in mice. *Neurogastroenterol Motil* 2005; **17**: 854-862

[PMID: 16336501 DOI: 10.1111/j.1365-2982.2005.00710.x]
124 **Jones RC**, Xu L, Gebhart GF. The mechanosensitivity of mouse colon afferent fibers and their sensitization by inflammatory mediators require transient receptor potential vanilloid 1 and acid-sensing ion channel 3. *J Neurosci* 2005; **25**: 10981-10989 [PMID: 16306411 DOI: 10.1523/JNEUROSCI.0703-05.2005]
125 **Blackshaw LA**, Brierley SM, Hughes PA. TRP channels: new targets for visceral pain. *Gut* 2010; **59**: 126-135 [PMID: 20007960 DOI: 10.1136/gut.2009.179523]
126 **Yiangou Y**, Facer P, Dyer NH, Chan CL, Knowles C, Williams NS, Anand P. Vanilloid receptor 1 immunoreactivity in inflamed human bowel. *Lancet* 2001; **357**: 1338-1339 [PMID: 11343743 DOI: 10.1016/S0140-673600)04503-7]
127 **Akbar A**, Yiangou Y, Facer P, Walters JR, Anand P, Ghosh S. Increased capsaicin receptor TRPV1-expressing sensory fibres in irritable bowel syndrome and their correlation with abdominal pain. *Gut* 2008; **57**: 923-929 [PMID: 18252749 DOI: 10.1136/gut.2007.138982]
128 **Bautista DM**, Movahed P, Hinman A, Axelsson HE, Sterner O, Högestätt ED, Julius D, Jordt SE, Zygmunt PM. Pungent products from garlic activate the sensory ion channel TRPA1. *Proc Natl Acad Sci USA* 2005; **102**: 12248-12252 [PMID: 16103371 DOI: 10.1073/pnas.0505356102]
129 **Namer B**, Kleggetveit IP, Handwerker H, Schmelz M, Jorum E. Role of TRPM8 and TRPA1 for cold allodynia in patients with cold injury. *Pain* 2008; **139**: 63-72 [PMID: 18440147 DOI: 10.1016/j.pain.2008.03.007]
130 **Brierley SM**, Hughes PA, Page AJ, Kwan KY, Martin CM, O'Donnell TA, Cooper NJ, Harrington AM, Adam B, Liebregts T, Holtmann G, Corey DP, Rychkov GY, Blackshaw LA. The ion channel TRPA1 is required for normal mechanosensation and is modulated by algesic stimuli. *Gastroenterology* 2009; **137**: 2084-2095.e3 [PMID: 19632231 DOI: 10.1053/j.gastro.2009.07.048]
131 **Kremeyer B**, Lopera F, Cox JJ, Momin A, Rugiero F, Marsh S, Woods CG, Jones NG, Paterson KJ, Fricker FR, Villegas A, Acosta N, Pineda-Trujillo NG, Ramírez JD, Zea J, Burley MW, Bedoya G, Bennett DL, Wood JN, Ruiz-Linares A. A gain-of-function mutation in TRPA1 causes familial episodic pain syndrome. *Neuron* 2010; **66**: 671-680 [PMID: 20547126 DOI: 10.1016/j.neuron.2010.04.030]
132 **Andrade EL**, Ferreira J, André E, Calixto JB. Contractile mechanisms coupled to TRPA1 receptor activation in rat urinary bladder. *Biochem Pharmacol* 2006; **72**: 104-114 [PMID: 16725114 DOI: 10.1016/j.bcp.2006.04.003]
133 **Akopian AN**. Regulation of nociceptive transmission at the periphery via TRPA1-TRPV1 interactions. *Curr Pharm Biotechnol* 2011; **12**: 89-94 [PMID: 20932255 DOI: 10.2174/138920111793937952]
134 **Vermeulen W**, De Man JG, De Schepper HU, Bult H, Moreels TG, Pelckmans PA, De Winter BY. Role of TRPV1 and TRPA1 in visceral hypersensitivity to colorectal distension during experimental colitis in rats. *Eur J Pharmacol* 2013; **698**: 404-412 [PMID: 23099257 DOI: 10.1016/j.ejphar.2012.10.014]
135 **Malykhina AP**, Qin C, Greenwood-van Meerveld B, Foreman RD, Lupu F, Akbarali HI. Hyperexcitability of convergent colon and bladder dorsal root ganglion neurons after colonic inflammation: mechanism for pelvic organ cross-talk. *Neurogastroenterol Motil* 2006; **18**: 936-948 [PMID: 16961697 DOI: 10.1111/j.1365-2982.2006.00807.x]
136 **Chitkara DK**, van Tilburg MA, Blois-Martin N, Whitehead WE. Early life risk factors that contribute to irritable bowel syndrome in adults: a systematic review. *Am J Gastroenterol* 2008; **103**: 765-774; quiz 775 [PMID: 18177446 DOI: 10.1111/j.1572-0241.2007.01722.x]
137 **Brumovsky PR**, Gebhart GF. Visceral organ cross-sensitization - an integrated perspective. *Auton Neurosci* 2010; **153**: 106-115 [PMID: 19679518 DOI: 10.1016/j.autneu.2009.07.006]
138 **Agostini A**, Filippini N, Cevolani D, Agati R, Leoni C, Tambasco R, Calabrese C, Rizzello F, Gionchetti P, Ercolani M, Leonardi M, Campieri M. Brain functional changes in patients with ulcerative colitis: a functional magnetic resonance imaging study on emotional processing. *Inflamm Bowel Dis* 2011; **17**: 1769-1777 [PMID: 21744432 DOI: 10.1002/ibd.21549]
139 **Coffin B**, Bouhassira D, Sabaté JM, Barbe L, Jian R. Alteration of the spinal modulation of nociceptive processing in patients with irritable bowel syndrome. *Gut* 2004; **53**: 1465-1470 [PMID: 15361496 DOI: 10.1136/gut.2003.031310]
140 **Mayer EA**, Aziz Q, Coen S, Kern M, Labus JS, Lane R, Kuo B, Naliboff B, Tracey I. Brain imaging approaches to the study of functional GI disorders: a Rome working team report. *Neurogastroenterol Motil* 2009; **21**: 579-596 [PMID: 19646070 DOI: 10.1111/j.1365-2982.2009.01304.x]
141 **Van Oudenhove L**, Coen SJ, Aziz Q. Functional brain imaging of gastrointestinal sensation in health and disease. *World J Gastroenterol* 2007; **13**: 3438-3445 [PMID: 17659690]
142 **Smith JK**, Humes DJ, Head KE, Bush D, White TP, Stevenson CM, Brookes MJ, Marciani L, Spiller RC, Gowland PA, Francis ST. fMRI and MEG analysis of visceral pain in healthy volunteers. *Neurogastroenterol Motil* 2011; **23**: 648-e260 [PMID: 21507149 DOI: 10.1111/j.1365-2982.2011.01712.x]
143 **Woolf CJ**. Central sensitization: implications for the diagnosis and treatment of pain. *Pain* 2011; **152**: S2-15 [PMID: 20961685 DOI: 10.1016/j.pain.2010.09.030]
144 **Petrenko AB**, Yamakura T, Baba H, Shimoji K. The role of N-methyl-D-aspartate (NMDA) receptors in pain: a review. *Anesth Analg* 2003; **97**: 1108-1116 [PMID: 14500166 DOI: 10.1213/01.ANE.0000081061.12235.55]
145 **Galan A**, Laird JM, Cervero F. In vivo recruitment by painful stimuli of AMPA receptor subunits to the plasma membrane of spinal cord neurons. *Pain* 2004; **112**: 315-323 [PMID: 15561387 DOI: 10.1016/j.pain.2004.09.011]
146 **Lin Y**, Roman K, Foust KD, Kaspar BK, Bailey MT, Stephens RL. Glutamate Transporter GLT-1 Upregulation Attenuates Visceral Nociception and Hyperalgesia via Spinal Mechanisms Not Related to Anti-Inflammatory or Probiotic Effects. *Pain Res Treat* 2011; **2011**: 507029 [PMID: 22220274 DOI: 10.1155/2011/507029]
147 **Woolf CJ**, Salter MW. Neuronal plasticity: increasing the gain in pain. *Science* 2000; **288**: 1765-1769 [PMID: 10846153 DOI: 10.1126/science.288.5472.1765]
148 **Reinold H**, Ahmadi S, Depner UB, Layh B, Heindl C, Hamza M, Pahl A, Brune K, Narumiya S, Müller U, Zeilhofer HU. Spinal inflammatory hyperalgesia is mediated by prostaglandin E receptors of the EP2 subtype. *J Clin Invest* 2005; **115**: 673-679 [PMID: 15719070]
149 **Cao J**, Yang X, Liu YN, Suo ZW, Shi L, Zheng CR, Yang HB, Li S, Hu XD. GABAergic disinhibition induced pain hypersensitivity by upregulating NMDA receptor functions in spinal dorsal horn. *Neuropharmacology* 2011; **60**: 921-929 [PMID: 21277878 DOI: 10.1016/j.neuropharm.2011.01.034]
150 **Samad TA**, Moore KA, Sapirstein A, Billet S, Allchorne A, Poole S, Bonventre JV, Woolf CJ. Interleukin-1beta-mediated induction of Cox-2 in the CNS contributes to inflammatory pain hypersensitivity. *Nature* 2001; **410**: 471-475 [PMID: 11260714 DOI: 10.1038/35068566]
151 **Argoff C**. Mechanisms of pain transmission and pharmacologic management. *Curr Med Res Opin* 2011; **27**: 2019-2031 [PMID: 21916528 DOI: 10.1185/03007995.2011.614934]
152 **Bradesi S**. Role of spinal cord glia in the central processing of peripheral pain perception. *Neurogastroenterol Motil* 2010; **22**: 499-511 [PMID: 20236247 DOI: 10.1111/j.1365-2982.2010.01491.x]
153 **Al-Chaer ED**, Kawasaki M, Pasricha PJ. A new model of chronic visceral hypersensitivity in adult rats induced by colon irritation during postnatal development. *Gastroenterology* 2000; **119**: 1276-1285 [PMID: 11054385 DOI: 10.1053/gast.2000.19576]

154 **Harrington AM**, Brierley SM, Isaacs N, Hughes PA, Castro J, Blackshaw LA. Sprouting of colonic afferent central terminals and increased spinal mitogen-activated protein kinase expression in a mouse model of chronic visceral hypersensitivity. *J Comp Neurol* 2012; **520**: 2241-2255 [PMID: 22237807 DOI: 10.1002/cne.23042]

155 **Gao J**, Wu X, Owyang C, Li Y. Enhanced responses of the anterior cingulate cortex neurones to colonic distension in viscerally hypersensitive rats. *J Physiol* 2006; **570**: 169-183 [PMID: 16239277 DOI: 10.1113/jphysiol.2005.096073]

156 **Azpiroz F**. Gastrointestinal perception: pathophysiological implications. *Neurogastroenterol Motil* 2002; **14**: 229-239 [PMID: 12061907 DOI: 10.1046/j.1365-2982.2002.00324.x]

157 **Bulmer DC**, Grundy D. Achieving translation in models of visceral pain. *Curr Opin Pharmacol* 2011; **11**: 575-581 [PMID: 22000605 DOI: 10.1016/j.coph.2011.09.008]

158 **Rao SS**, Read NW, Davison PA, Bannister JJ, Holdsworth CD. Anorectal sensitivity and responses to rectal distention in patients with ulcerative colitis. *Gastroenterology* 1987; **93**: 1270-1275 [PMID: 3678745]

159 **Faure C**, Giguère L. Functional gastrointestinal disorders and visceral hypersensitivity in children and adolescents suffering from Crohn's disease. *Inflamm Bowel Dis* 2008; **14**: 1569-1574 [PMID: 18521915 DOI: 10.1002/ibd.20506]

160 **Vergnolle N**. Postinflammatory visceral sensitivity and pain mechanisms. *Neurogastroenterol Motil* 2008; **20** Suppl 1: 73-80 [PMID: 18402644 DOI: 10.1111/j.1365-2982.2008.01110.x]

161 **Tillisch K**, Labus JS. Advances in imaging the brain-gut axis: functional gastrointestinal disorders. *Gastroenterology* 2011; **140**: 407-411.e1 [PMID: 21167161 DOI: 10.1053/j.gastro.2010.12.014]

162 **Larsson MB**, Tillisch K, Craig AD, Engström M, Labus J, Naliboff B, Lundberg P, Ström M, Mayer EA, Walter SA. Brain responses to visceral stimuli reflect visceral sensitivity thresholds in patients with irritable bowel syndrome. *Gastroenterology* 2012; **142**: 463-472.e3 [PMID: 22108191 DOI: 10.1053/j.gastro.2011.11.022]

163 **Wu F**, Zikusoka M, Trindade A, Dassopoulos T, Harris ML, Bayless TM, Brant SR, Chakravarti S, Kwon JH. MicroRNAs are differentially expressed in ulcerative colitis and alter expression of macrophage inflammatory peptide-2 alpha. *Gastroenterology* 2008; **135**: 1624-1635.e24 [PMID: 18835392 DOI: 10.1053/j.gastro.2008.07.068]

164 **Zhou Q**, Souba WW, Croce CM, Verne GN. MicroRNA-29a regulates intestinal membrane permeability in patients with irritable bowel syndrome. *Gut* 2010; **59**: 775-784 [PMID: 19951903 DOI: 10.1136/gut.2009.181834]

165 **Brain O**, Owens BM, Pichulik T, Allan P, Khatamzas E, Leslie A, Steevels T, Sharma S, Mayer A, Catuneanu AM, Morton V, Sun MY, Jewell D, Coccia M, Harrison O, Maloy K, Schönefeldt S, Bornschein S, Liston A, Simmons A. The intracellular sensor NOD2 induces microRNA-29 expression in human dendritic cells to limit IL-23 release. *Immunity* 2013; **39**: 521-536 [PMID: 24054330 DOI: 10.1016/j.immuni.2013.08.035]

166 **Tran L**, Chaloner A, Sawalha AH, Greenwood Van-Meerveld B. Importance of epigenetic mechanisms in visceral pain induced by chronic water avoidance stress. *Psychoneuroendocrinology* 2013; **38**: 898-906 [PMID: 23084728 DOI: 10.1016/j.psyneuen.2012.09.016]

167 **Sharma A**, Lelic D, Brock C, Paine P, Aziz Q. New technologies to investigate the brain-gut axis. *World J Gastroenterol* 2009; **15**: 182-191 [PMID: 19132768 DOI: 10.3748/wjg.15.182]

168 **Pellissier S**, Dantzer C, Canini F, Mathieu N, Bonaz B. Psychological adjustment and autonomic disturbances in inflammatory bowel diseases and irritable bowel syndrome. *Psychoneuroendocrinology* 2010; **35**: 653-662 [PMID: 19910123 DOI: 10.1016/j.psyneuen.2009.10.004]

169 **Wilder-Smith CH**. The balancing act: endogenous modulation of pain in functional gastrointestinal disorders. *Gut* 2011; **60**: 1589-1599 [PMID: 21768212 DOI: 10.1136/gutjnl-2011-300253]

P- Reviewer: Wiley JW **S- Editor**: Zhai HH **L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.1021

World J Gastroenterol 2014 January 28; 20(4): 1021-1029
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Portal inflow preservation during portal diversion in small-for-size syndrome

Xian-Qiang Wang, Yue-Fang Xu, Jing-Wang Tan, Wen-Ping Lv, Zhe Liu, Jian-Ping Zeng, Jia-Hong Dong

Xian-Qiang Wang, Department of Hepatobiliary Surgery, Qilu Hospital, Shandong University, Jinan 250012, China
Xian-Qiang Wang, Wen-Ping Lv, Zhe Liu, Jian-Ping Zeng, Jia-Hong Dong, Department of Hepatobiliary Surgery, the Chinese PLA General Hospital, Beijing 100853, China
Yue-Fang Xu, Department of Pharmacology, First Hospital Affiliated to the Chinese PLA General Hospital, Beijing 100048, China
Jing-Wang Tan, Department of Hepatobiliary Surgery, First Hospital Affiliated to the Chinese PLA General Hospital, Beijing 100048, China
Author contributions: Wang XQ and Dong JH conceived and designed the experiments; Wang XQ, Tan JW and Xu YF performed the experiments; Lv WP, Liu Z and Zeng JP analyzed the data; Wang XQ and Tan JW wrote the paper; all the authors have read and approved the final manuscript to be published.
Supported by Chinese Ministry of Health, China, No. 200802012, the Project of National Natural Science Foundation of China, No. 81271738; and the National Science and Technology Major Project, No. 2012BAI06B01
Correspondence to: Jia-Hong Dong, MD, PhD, FACS, Department of Hepatobiliary Surgery, the Chinese PLA General Hospital, Beijing, China. dongjh_301@163.com
Telephone: +86-10-66938030 Fax: +86-10-66848636
Received: April 3, 2013 Revised: July 23, 2013
Accepted: September 4, 2013
Published online: January 28, 2014

Abstract

AIM: To investigate the impact of portal inflow on liver remnants in a stable pig model of small-for-size syndrome.

METHODS: Twenty pigs underwent mesocaval shunt (MCS) surgery followed by 85%-90% hepatectomy. The control group had no shunt placement; the S_1 group had portal flow maintained at an average of 2.0 times the baseline values; and the S_2 group had portal flow maintained at an average of 3.2 times the baseline flow. The effect of portal functional competition on the liver remnant was investigated for 48 h postoperatively. Data were presented as mean ± SD. Statistical significance was determined using Student's *t* test (SPSS, Chicago, IL, United States). Values of $P < 0.05$ were considered statistically significant.

RESULTS: At 24 h after hepatectomy, biochemical and histological changes were not significantly different between the S_1 and S_2 groups, but changes in both sets of variables were significantly less than in the control group. At 48 h, biochemical and histological changes were significantly less in the S_2 group than in the S_1 or control group. The regeneration index was significantly higher in the S_2 group than in the S_1 group, and was similar to that in the control group. Apoptosis index, serum lipopolysaccharide, and bacterial DNA levels were significantly lower in the S_2 group than in the other two groups.

CONCLUSION: Diversion of portal inflow using MCS reduces portal overflow injury. Excessive diversion of portal inflow inhibits liver regeneration following major hepatectomy. Maintaining portal inflow at an average of 3.2 times above baseline helps promote hypertrophy of the liver remnant and reduce apoptosis.

Key words: Portal flow; Portal diversion; Small-for-size syndrome; Mesocaval shunt

Core tip: We established a model of small-for-size syndrome in pigs undergoing 85%-90% hepatectomy with mesocaval shunt (MCS) placement to define the optimal portal inflow required to preserve liver regeneration. Our findings indicate that diversion of portal inflow by MCS reduces injury from portal overflow following major hepatectomy, whereas excessive diversion of portal flow can retard liver regeneration. Preservation of portal inflow to at least 3.2 times above baseline levels appeared to promote hepatocyte hypertrophy and reduce apoptosis.

Wang XQ, Xu YF, Tan JW, Lv WP, Liu Z, Zeng JP, Dong JH. Portal inflow preservation during portal diversion in small-for-size syndrome. *World J Gastroenterol* 2014; 20(4): 1021-1029 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/1021.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.1021

INTRODUCTION

Major hepatectomy with partial graft transplantation causes simultaneous death and regeneration of hepatocytes. Small-for-size syndrome (SFSS) develops following this procedure if the functional liver mass is inadequate to maintain a balance between regeneration and metabolic demands[1-4]. Portal venous hypoperfusion of an extremely small residual liver or partial liver allograft is considered to be one of the most important factors leading to dysfunction following hepatectomy[5]. Portal diversion to the vena cava, using a mesocaval shunt (MCS) or portocaval shunt (PCS), is used to relieve portal hypoperfusion in both experimental and clinical settings[6-10]. However, the functional competition that occurs between the portal vein and systemic circulation, and its impact on the liver remnant have yet to be investigated.

In this study, we established a model of SFSS in pigs undergoing 85%-90% hepatectomy with MCS placement. Portal vein inflow (PVF) was regulated by modulating the size of the MCS. The study was undertaken to define the optimal portal inflow required to preserve liver regeneration.

MATERIALS AND METHODS

Experimental animals

Twenty-five male Bama miniature pigs (15-20 kg), aged 4-6 mo were obtained from the Pig and Poultry Production Institute (Guangxi Province, China). The pigs were raised from a closed herd and kept under strict quarantine. All experiments were conducted in accordance with Chinese legislation on protection of animals and complied with the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985). The study was approved by the Animal Care and Use Committee and the Ethics Committee of the Chinese People's Liberation Army General Hospital. Every effort was made to minimize any suffering of the animals used in this study.

Surgical procedures

The pigs were deprived of food for 8 h before the operation. Initial sedation was achieved with a deep intramuscular injection of ketamine (15-20 mg/kg) and chlorpromazine (6-8 mg/kg), which were administered 15 min after atropine (0.01 mg/kg). Oxygen saturation and heart rate were monitored throughout the operation, and anesthesia was maintained using 1.5% halothane in oxygen titrated to provide anesthesia.

Central venous access was established using a catheter in the right femoral vein. Normal saline (1 L) and 5% dextrose (500 mL) were administered intravenously during the surgical procedure. No attempt was made to lower central venous pressure.

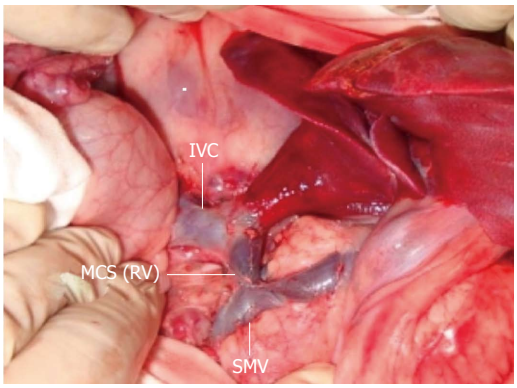


Figure 1 Photograph of the vascular anastomosis with the renal vein. IVC: Inferior vena cava; RV: Renal vein; MCS: Mesocaval shunt; SMV: Superior mesenteric vein.

An upper-midline incision was made, and a 16-gauge catheter was inserted into the main portal vein *via* the gastroduodenal vein to measure portal vein pressure (PVP). Two ultrasonic probes (TS420; Transonic Systems, Ithaca, NY, United States) were used to assist the laparotomy. A 9-mm diameter probe was placed around the main portal vein (downstream of the gastroduodenal vein), and a 3.5-mm probe was placed around the hepatic artery near its origin from the celiac artery. The origin of the hepatic artery was isolated by ligation of the right gastric and gastroduodenal arteries. MCSs with different anastomotic diameters (5-10 mm) were implanted. Left trilobectomy was performed, together with partial right-posterior-lobe resection, without hepatic pedicle occlusion[11]. Parts of the right posterior and caudate lobes were retained to leave a residual hepatic volume of 10%-15% of the normal liver volume.

The mesenteric venous inflow was diverted through an MCS constructed using the prepared left renal vein with the PVF partly occluded (Figure 1). MCS, PVF and hepatic artery flow (HAF) were measured before and 30 min after MCS implantation. If the portal vein inflow was > 3.5 times higher than baseline or if shunt occlusion occurred, the shunt was closed, and the pigs were assigned to the control group (n = 6). Measurement of portal flow was repeated 10 min later. If the portal flow was < 1.8 times the baseline value, the shunt was adjusted to increase the portal flow to 1.8-2.3 or 3.0-3.5 times the baseline value. If necessary, an empty balloon with a catheter was placed around the shunt so that blood flow could be regulated by expanding the balloon. Animals with a portal flow 1.8-2.3 times the baseline value were assigned to the S_1 group (n = 7). Animals with a portal flow 3.0-3.5 times the baseline value were assigned to the S_2 group (n = 7). Five animals were excluded from the study because of shunt obliteration or other surgical complications during the observation period.

Forty-eight hours after hepatectomy, the animals were

reopened. PVP, PVF and HAF were recorded and blood and tissue samples were obtained. Local anesthetic (50 mg marcaine in 20 mL) was administered subcutaneously to the abdominal wound. Halothane was discontinued postoperatively and a single dose of 375 mg penicillin was given intramuscularly to all pigs. Normal saline (500 mL) and 10% glucose solution (500 mL) were administered during recovery and daily thereafter.

The pigs were monitored until 48 h after hepatectomy, when they were anesthetized and reopened before euthanasia. Injury to the sinusoidal endothelial cells, dynamic PVF and HAF, injury and regeneration of the liver remnant, serum endotoxin levels, and bacterial translocation were compared between the three groups. At the end of the experiments, the pigs were sacrificed by an overdose of potassium chloride.

Blood and serum analysis

Serial serum samples were collected during the follow-up period. Blood sampling was performed preoperatively, at 2 h after hepatectomy, then daily until euthanasia. Serum levels of alanine aminotransferase (ALT), total bilirubin (TB), international normalized ratio (INR), hyaluronic acid (HA), and thymidine kinase (TK) activity were determined. HA levels were monitored to reflect the degree of sinusoidal endothelial damage[12,13]. Values were determined using the Pharmacia HA radiometric assay kit (Shanghai Yi Hua Scientific, Inc., China). TK activity was used as an index of hepatic regeneration[14] and was measured in serial serum samples using the Liaison TK assay. Results were expressed as dpm/mL protein (Jingmei Biotech Co. Ltd., Shenzhen, China).

Tissue analysis

Hepatic tissue was sampled in the three groups at 48 h after hepatectomy. Each biopsy sample was divided into two sections. One was immediately cut into 1-mm cubes and fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 mol/L sodium cacodylate-HCl, pH 7.4) overnight at 4 ℃ prior to sectioning for transmission electron microscopy (TEM). The other section was preserved in 10% formaldehyde prior to embedding in paraffin. The tissue samples were sectioned and stained with hematoxylin and eosin (HE) using standard histological techniques.

The pigs were sacrificed at 48 h after hepatectomy, and the patency of the MCS was verified surgically. The liver was excised, weighed and processed. Hepatic tissue was sampled for proliferating cell nuclear antigen (PCNA) staining and *in situ* terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL).

For histology and morphometry, 4-μm-thick sections, prepared from formalin-fixed, paraffin-embedded liver tissues, were stained with hematoxylin-phloxin-saffron and periodic acid Schiff staining. PCNA expression was detected by immunostaining using a monoclonal anti-PCNA-antibody kit (Jingmei Biotech). In addition, 3-μm sections were stained *in situ* with TUNEL using an apoptosis *in situ* detection kit (*Jingmei Biotech* Co., Ltd, Shenzhen, China) according to the manufacturer's instructions.

Hepatic regeneration and apoptosis

Increases in liver volume and PCNA index (PI) were used to quantify hepatic regeneration. The rate of increase in liver volume after hepatectomy was evaluated as: regenerated liver volume at sacrifice/estimated remnant liver volume at operation × 100%. PCNA data were expressed as the percentage of PCNA-stained hepatocytes per total number of hepatocytes (PI). The percentage of TUNEL-positive cells relative to the total cell count was used to estimate the apoptosis index (AI). Counts were made in 10 high-power fields for each of the three groups.

Lipopolysaccharide and bacterial translocation

Lipopolysaccharide (LPS) levels were quantitated using the limulus amebocyte lysate (LAL) assay, which is based on the methods introduced by Iwanaga and colleagues[15]. The assay was performed using a commercially available chromogenic LAL endpoint QCL 1000 Kit (Yihua Bio-Science, Shanghai, China) following the manufacturer's instructions. Standards and samples were analyzed in duplicate.

Real-time polymerase chain reaction assay for total bacterial quantification

DNA was extracted from blood using the Fast DNA Spin Kit (Qiagen, Valencia, CA, United States) according to the manufacturer's instructions. Total bacterial quantification was performed using 16S rRNA-gene-targeted primers. The universal primers were 5'-TTCCGGTTGATCCTGCCGGA-3' forward, 5'-GGTTACCTTGTTACGACTT-3' reverse[16,17].

Real-time polymerase chain reaction (PCR) was performed on an iCycler IQ real-time detection system coupled to iCycler optical system interface version 2.3 software (Bio-Rad, Veenendaal, Netherlands). Serially diluted genomic DNA from selected bacterial isolates was used as a real-time PCR control for total bacterial quantification. PCR bacterial counts were expressed as $\log_{10}$ cells/g tissue ± SE.

Statistical analysis

Data were presented as mean ± SD. Statistical significance was determined using Student's *t* test (SPSS, Chicago, IL, United States). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Operative characteristics

The operative characteristics are shown in Table 1. There were no significant differences among the three groups ($P > 0.05$).

Hemodynamic studies

Systemic arterial pressure was monitored throughout the study. Serial changes in PVF and HAF are shown in Table 2. At baseline, PVF, HAF and PVP in the three

Table 1 Operative characteristics

	Control group	S_1 group	S_2 group	*P* value[1]	*P* value[2]
Body weight (kg)	17.8 ± 3.1	18.1 ± 2.9	18.5 ± 3.9	0.85	0.87
Left trilobes (g)	351.2 ± 14.9	357.5 ± 17.2	365.5 ± 15.8	0.89	0.91
ETL (g)	443.1 ± 18.8	446.9 ± 21.5	457.0 ± 19.8	0.77	0.86
WRL (g)	391.8 ± 19.4	389.8 ± 17.4	400.8 ± 21.4	0.95	0.92
ERL(g)	51.3 ± 6.8	57.1 ± 8.5	56.2 ± 7.1	0.89	0.84
Proportion of ERL	11.8% ± 2.3%	12.8% ± 3.3%	12.2% ± 3.5%	0.87	0.83

Control group, no shunt placement; S_1 group, portal flow maintained at an average 2.0 times baseline values; S_2 group, portal flow maintained at an average 3.2 times baseline flow. Data expressed as mean ± SD. Estimated total liver volume (ETL) = (weight of left trilobes) × 100/80; WRL: Weight of resected liver; ERL: Estimated residual liver volume. [1]The difference between the control group and S_2 group; [2]The difference between S_1 and S_2 groups.

Table 2 Changes in portal vein inflow and hepatic artery flow at baseline, 24 and 48 h after hepatectomy, and at euthanasia in the three groups of animals

	Control group	S_1 group	S_2 group	*P* value[1]	*P* value[2]
PVF, L/min per 100 g					
BAS	59.4 ± 11.4	62.1 ± 11.4	67.4 ± 11.6	0.840	0.780
PH	451.8 ± 31.1	146.8 ± 21.1	218.8 ± 29.3	0.000	0.001
EUT	220.3 ± 41.3	69.8 ± 18.6	125.3 ± 31.6	0.000	0.000
HAF, mL/min per 100 g					
BAS	19.4 ± 4.5	18.3 ± 3.4	19.9 ± 4.1	0.920	0.910
PH	6.1 ± 2.5	12.1 ± 3.5	14.9 ± 2.5	0.001	0.061
EUT	5.5 ± 2.1	11.1 ± 3.4	13.2 ± 4.2	0.000	0.052
P/A					
BAS	3.1 ± 0.2	3.4 ± 0.3	3.4 ± 0.2	0.780	0.940
PH	74.0 ± 8.1	12.1 ± 2.8	14.8 ± 3.1	0.001	0.040
EUT	40.8 ± 6.6	6.3 ± 1.2	9.5 ± 1.8	0.000	0.001
PVP					
BAS	6.4 ± 1.8	6.9 ± 1.3	6.0 ± 0.8	0.930	0.750
PH	13.8 ± 2.6	7.6 ± 1.6	8.7 ± 1.4	0.022	0.061
EUT	15.9 ± 2.5	8.9 ± 1.2	9.6 ± 1.5	0.001	0.042

All flow values are reported in mL/min per 100 g hepatic tissue. BAS: Baseline; EUT: Euthanasia; NS: Not significant; P/A: Portal-to-arterial flow ratio; PVF: Portal vein inflow; HAF: Hepatic artery flow. [1]The difference between the control group and S_2 group; [2]The difference between the S_1 and S_2 groups.

groups did not differ significantly. However, at 24 or 48 h after hepatectomy, PVF and portal-to-arterial flow ratio in the S_2 group were significantly lower than in the control group, and significantly higher than in the S_1 group. HAF in the S_2 group was significantly higher than in the control group, and did not differ significantly from that in the S_1 group. PVP in the S_2 group was significantly lower than in the control group, and did not differ significantly compared with the S_1 group.

Hepatocellular injury

Preoperative and serial postoperative measurements of serum ALT, TB, and INR are shown in Figure 2. During the first 24 h after hepatectomy, all parameters except ALT were significantly higher in the control group than in the S_1 group. There were no significant differences in TB or INR between the groups. However, at 48 h after hepatectomy, serum ALT, TB and INR were significantly lower in the S_2 group than in the S_1 and control groups.

Sinusoidal endothelial injury

In the control group, there was no portal diversion. Both HE and TEM examination showed significant endothelial injury, accompanied by sinusoidal dilation, hydropic changes in hepatocytes and hemorrhage in the hepatic parenchyma (Figure 3A and C). In the S_1 and S_2 groups there was only mild sinusoidal injury to the hepatic microarchitecture and no intraparenchymal hemorrhage was seen (Figure 3B and D). Serial changes in HA levels in the three groups are shown in Figure 4. Following 85%-90% hepatectomy, serum HA levels increased in all three groups. At 2 h after hepatectomy, HA levels were significantly higher in the control group than in the S_1 or S_2 groups.

Liver regeneration and apoptosis

The rate of increase in the weight of the liver remnants was significantly higher in the S_2 group than in the control or S_1 groups. The rate of increase was lower in the S_1 group than in the control group (Figure 5A). There were also differences between the three groups with respect to the estimated PI in PCNA-stained tissue at 48 h PH (Figure 5B and C).

At 2 h after hepatectomy, TK activity was significantly higher in the control group than in the S_1 or S_2 groups (Figure 5D). TK levels in the S_2 group remained stable,

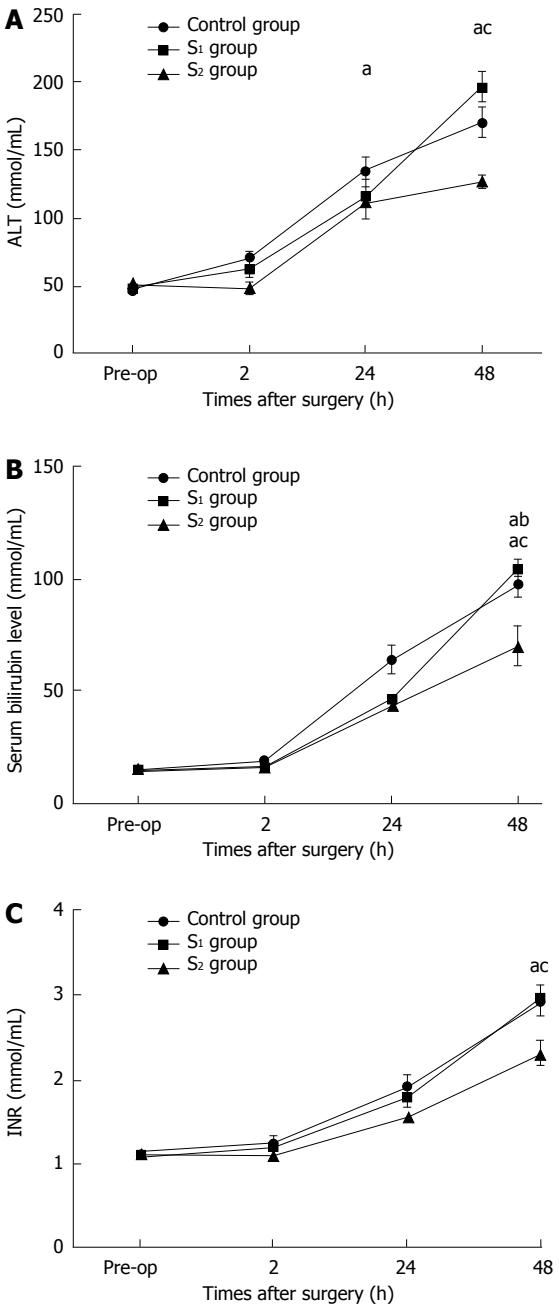


Figure 2 Changes in serum alanine aminotransferase, total bilirubin and international normalized ratio values. A: Alanine aminotransferase (ALT); B: Total bilirubin; C: International normalized ratio (INR). [a]$P < 0.05$ for comparison between S_1 and control groups; [c]$P < 0.05$ for comparison between S_2 and S_1 groups.

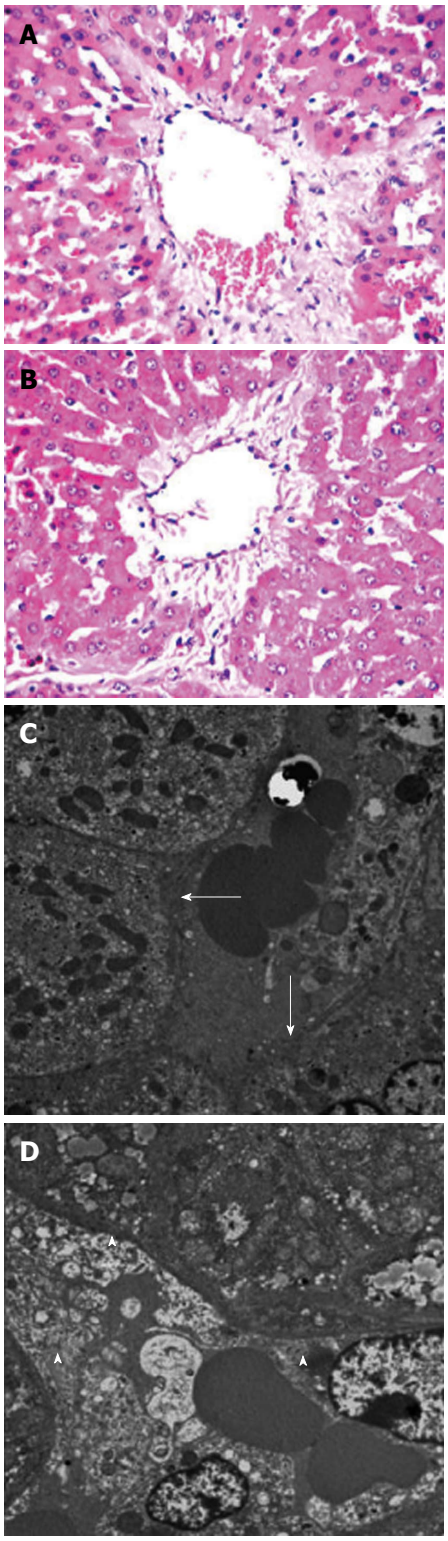


Figure 3 Sinusoidal endothelial injury following hepatectomy. A and B: Hematoxylin and eosin staining; C and D: Transmission electron microscopy of tissue samples taken 1 h after hepatectomy (× 400 magnification). Structure of the endothelial lining was preserved (arrow); Sinusoidal endothelial lining destroyed (arrow head).

and at 48 h after hepatectomy, they were significantly higher than in the S_1 group and comparable to those in the control group.

At 48 h after hepatectomy, there were high numbers of TUNEL-positive cells in the liver remnant (Figure 6A). The AI at 48 h after hepatectomy was significantly lower in the S_2 group than in the control group (Figure 6B).

DISCUSSION

Animal experiments have shown that hepatectomy decreases the size of the hepatic vascular bed and has the potential to increase portal pressure and vascular resistance, resulting in excessive portal flow and hemodynamic instability[3,4,18]. Similar findings have been reported

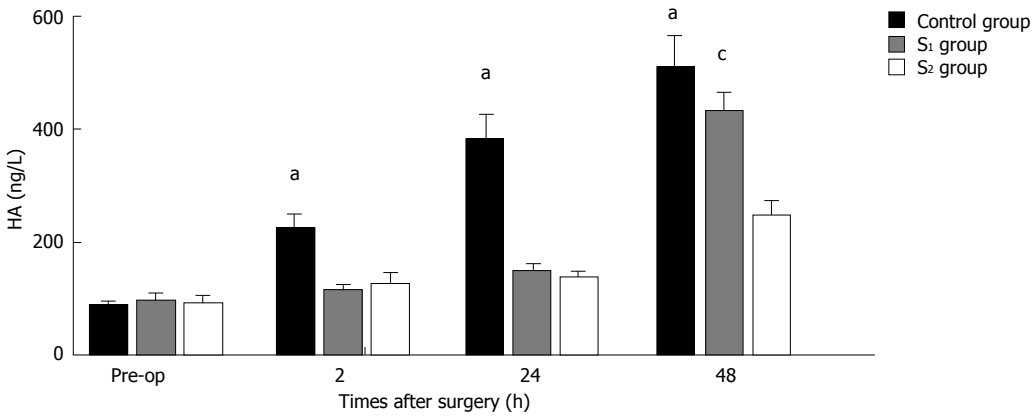


Figure 4 Serial changes in hyaluronic acid levels in the three groups. $^{a}P < 0.05$ for comparison between S_1 and control groups; $^{c}P < 0.05$ for comparison between S_2 and S_1 groups. HA: Hyaluronic acid.

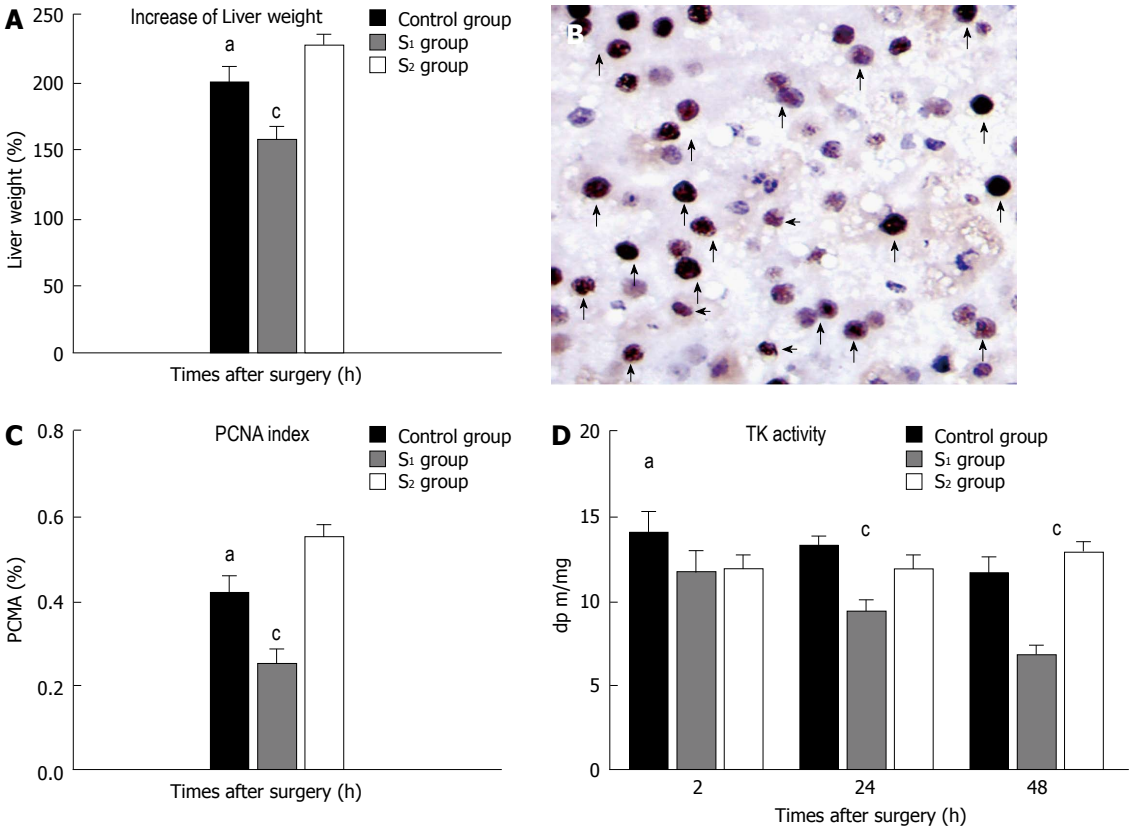


Figure 5 Liver regeneration and apoptosis. A: Percentage increase in liver remnant weights; B: Proliferating cell nuclear antigen (PCNA) staining of liver remnant (positive cells are indicated by arrows; × 400 magnification); C: Microphotometric evaluation of PCNA index in PCNA-stained tissue 48 h partial hepatectomy. Values are expressed as mean ± SD; $n = 6$ in both groups. D: Thymidine kinase (TK) activity. $^{a}P < 0.05$ for comparison between S_1 and control groups; $^{c}P < 0.05$ for comparison between S_2 and S_1 groups. PI was used to quantify hepatic regeneration. PCNA data were expressed as the percentage of PCNA-stained hepatocytes per total number of hepatocytes.

in clinical practice[7,8,19,20] and contribute to high postoperative morbidity and mortality rates[3,6,8]. Furthermore, severe damage to the sinusoidal endothelial cells of the remnant liver at 3 h postoperatively has been reported as one of the main factors responsible for the high mortality rates in dogs undergoing massive hepatectomy[21].

Many studies have shown that diversion of portal inflow, using PCS, or MCS and splenectomy, can relieve overflow injury and improve survival and prognosis[3,4,21-24]. Despite these encouraging results, the use of PCS is associated with a marked delay in liver regeneration[25,26]. This is thought to be the result of over-reduction of vascular shear stress in the portal vein, possibly accompanied by diversion of hepatotrophic factors into the systemic circulation. This technique may also lead to loss of portal flow between the liver remnant and system-

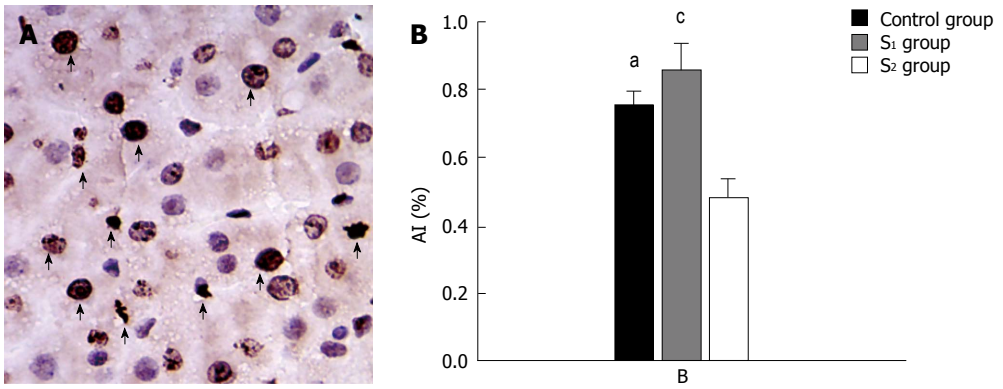


Figure 6 Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling staining and AI. A: Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay results at 48 h partial hepatectomy (× 400 magnification). Many TUNEL-positive cells (arrows) were present in the liver remnant; B: Microphotometric evaluation of apoptosis index (AI) in TUNEL-stained tissue at 48 h after hepatectomy. Values are expressed as mean ± SD. $^{a}P < 0.05$ for comparison between S_1 and control groups; $^{c}P < 0.05$ for comparison between S_2 and S_1 groups. Percentage of TUNEL-positive cells relative to the total cell count was used to estimate AI.

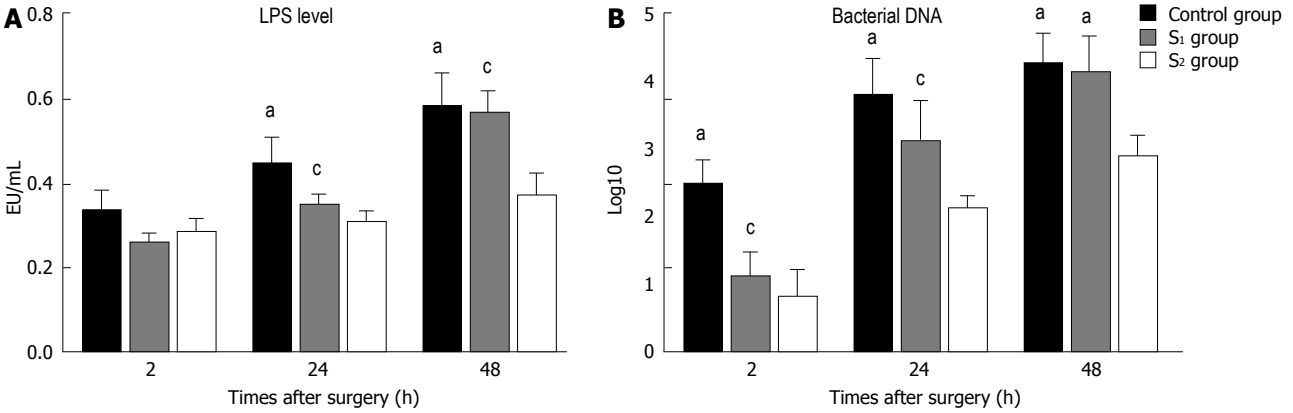


Figure 7 Serial changes in serum lipopolysaccharide levels (A) and blood bacterial DNA levels (B). $^{a}P < 0.05$ for comparison between S_1 and control groups; $^{c}P < 0.05$ for comparison between S_2 and S_1 groups. LPS: Lipopolysaccharide.

ic shunt. The problem is exacerbated as portal systemic pressure increases in the regenerating liver. To overcome these difficulties associated with MCS or PCS, sufficient portal inflow and pressure needs to be preserved to promote liver regeneration without injuring the sinusoidal endothelium.

The optimum portal inflow required to stimulate liver regeneration with minimal or no overflow injury to the liver remnant remains unknown. This is because opinions regarding the manageable upper limit of portal pressure differ between transplant centers. Workers in Japan[27] set the appropriate PVP at < 20 mmHg, whereas another study[24] recommended PVP < 15 mmHg for living donor liver transplantation (LDLT). Another group[28] reported that small left-lobe grafts with < 40% graft volume/standard liver volume can be used safely with a portal flow < 25 mmHg. In two other studies of LDLT[20,23], suitable cutoff values for portal inflow were reported to be 250 and 260 mL/min/100 g tissue.

A previous study in pigs[25] showed that it was necessary to maintain portal vein flow at approximately two times the baseline value in order to produce a favorable outcome. However, this study provided no information about the effects of portal functional competition on optimum portal inflow for the liver remnant.

In our study, we demonstrated that using an MCS in the S_1 and S_2 groups relieved sinusoidal endothelial injury relative to that seen in the control group with no shunt. Liver regeneration (determined by rate of growth and PI) in the S_2 group using a median portal inflow 3.2 times above baseline, was similar to that in the control group at 48 h after hepatectomy, and was significantly higher than in the S_1 group with a median portal inflow of 2.0 times baseline. The AI in the S_2 group was significantly lower than in the S_1 and control groups, indicating the portal inflow regimen used in the S_2 group supported liver regeneration and reduced apoptosis.

LPS levels indicated that the inflammatory response at 48 h after hepatectomy was less marked in the S_2 group than in the S_1 and control groups, further supporting the rationale for preserving > 3 times the baseline portal flow per unit tissue volume.

It has previously been demonstrated that competition between the portal vein and systemic circulation begins after a functional MCS has been established[20,23]. In our study the PVF per unit volume was lower in the S_1 and

S_2 groups than in the control group. In these groups, hypertrophy of the liver remnant resulted in an increase in vascular resistance per unit volume.

In the S_1 group, the PVF per unit volume decreased to the baseline value at 48 h after hepatectomy, whereas in the S_2 group, portal inflow remained twice that at baseline at the same time point (Table 2). These results indicate that preserving portal flow at twice the baseline level was insufficient to sustain hypertrophy of the liver remnant. However, preserving approximately 3.2 times the baseline portal flow resulted in a high growth rate and a PI similar to that in the control group.

Portal overflow injury, LPS/bacterial translocation, and inflammatory responses represent an important mechanism of pathogenesis. The liver contains reticuloendothelial cells (Kupffer cells), and it has been shown that function of the reticuloendothelial system decreases significantly after major hepatectomy[29,30]. Innate immunity is also significantly impaired following major liver resection[26,31], and portal hypertension has been shown to increase LPS absorption and bacterial translocation and cause severe inflammation[31,32]. In our study the marked LPS/bacterial translocation and inflammation responses seen in the control and S_1 groups delayed liver regeneration and aggravated apoptosis and injury to the liver remnant (Figure 7). These responses were far less marked in the S_2 group.

Taken together our findings indicate that diversion of portal inflow by MCS reduces injury from portal overflow following major hepatectomy, whereas excessive diversion of portal flow can retard liver regeneration. Preservation of portal inflow to at least 3.2 times above baseline levels appeared to promote hepatocyte hypertrophy and reduce apoptosis.

COMMENTS

Background

Excessive diversion of portal inflow associated with mesocaval shunts (MCS) in 'small-for-size' syndrome (SFSS) has the potential to retard liver regeneration. However, it is unclear the optimal portal inflow is required to preserved liver regeneration. This study investigated the impact of portal inflow on liver remnants in a stable pig model of SFSS.

Research frontiers

Portal diversion to the vena cava, using a MCS or portocaval shunt, is used to relieve portal hypoperfusion in both experimental and clinical settings. The functional competition between the portal vein and systemic circulation occurred, which may have an impact on the liver remnant.

Innovations and breakthroughs

This is the first study focusing on the impact of portal inflow on liver remnants in a stable pig model of SFSS. The authors demonstrated that diversion of portal inflow using MCS reduces portal overflow injury. Excessive diversion of portal inflow inhibits liver regeneration following major hepatectomy. Maintenance of portal inflow to at an average of 3.2 times above baseline levels appeared to promote hepatocyte hypertrophy and reduce apoptosis.

Applications

The results of this study provide some evidence that regulation of portal inflow is useful for patient following major hepatectomy to avoid SFSS.

Peer review

This study demonstrated that maintenance of portal inflow to at an average of 3.2 times above baseline levels appeared to promote hepatocyte hypertrophy and reduce apoptosis in a stable pig model of SFSS. Therefore, measures should be considered to modulate the portal inflow when the risk of SFSS in liver transplantation or extended hepatectomy is high.

REFERENCES

1 **Lee SS**, Hadengue A, Girod C, Braillon A, Lebrec D. Reduction of intrahepatic vascular space in the pathogenesis of portal hypertension. In vitro and in vivo studies in the rat. *Gastroenterology* 1987; **93**: 157-161 [PMID: 3582902]

2 **Dahm F**, Georgiev P, Clavien PA. Small-for-size syndrome after partial liver transplantation: definition, mechanisms of disease and clinical implications. *Am J Transplant* 2005; **5**: 2605-2610 [PMID: 16212618 DOI: 10.1111/j.1600-6143.2005.01081.x]

3 **Kanematsu T**, Takenaka K, Furuta T, Ezaki T, Sugimachi K, Inokuchi K. Acute portal hypertension associated with liver resection. Analysis of early postoperative death. *Arch Surg* 1985; **120**: 1303-1305 [PMID: 4051735 DOI: 10.1001/archsurg.1985.01390350083017]

4 **Kiuchi T**, Kasahara M, Uryuhara K, Inomata Y, Uemoto S, Asonuma K, Egawa H, Fujita S, Hayashi M, Tanaka K. Impact of graft size mismatching on graft prognosis in liver transplantation from living donors. *Transplantation* 1999; **67**: 321-327 [PMID: 10075602 DOI: 10.1097/00007890-199901270-00024]

5 **Fondevila C**, Hessheimer AJ, Taurá P, Sánchez O, Calatayud D, de Riva N, Muñoz J, Fuster J, Rimola A, García-Valdecasas JC. Portal hyperperfusion: mechanism of injury and stimulus for regeneration in porcine small-for-size transplantation. *Liver Transpl* 2010; **16**: 364-374 [PMID: 20209596 DOI: 10.1002/lt.21989]

6 **Wang H**, Ohkohchi N, Enomoto Y, Usuda M, Miyagi S, Masuoka H, Sekiguchi S, Kawagishi N, Fujimori K, Sato A, Satomi S. Effect of portocaval shunt on residual extreme small liver after extended hepatectomy in porcine. *World J Surg* 2006; **30**: 2014-2022; discussion 2023-2024 [PMID: 16927066]

7 **Lan AK**, Luk HN, Goto S, Chen SM, Eng HL, Chen YS, de Villa VH, Wang CC, Cheng YF, Chen CL, Lee JH, Jawan B. Stress response to hepatectomy in patients with a healthy or a diseased liver. *World J Surg* 2003; **27**: 761-764 [PMID: 14509501 DOI: 10.1007/s00268-003-6955-2]

8 **Troisi R**, Ricciardi S, Smeets P, Petrovic M, Van Maele G, Colle I, Van Vlierberghe H, de Hemptinne B. Effects of hemiportocaval shunts for inflow modulation on the outcome of small-for-size grafts in living donor liver transplantation. *Am J Transplant* 2005; **5**: 1397-1404 [PMID: 15888047 DOI: 10.1111/j.1600-6143.2005.00850.x]

9 **Suehiro T**, Shimada M, Kishikawa K, Shimura T, Soejima Y, Yoshizumi T, Hashimoto K, Mochida Y, Hashimoto S, Maehara Y, Kuwano H. Effect of intraportal infusion to improve small for size graft injury in living donor adult liver transplantation. *Transpl Int* 2005; **18**: 923-928 [PMID: 16008741 DOI: 10.1111/j.1432-2277.2005.00159.x]

10 **Yamada T**, Tanaka K, Uryuhara K, Ito K, Takada Y, Uemoto S. Selective hemi-portocaval shunt based on portal vein pressure for small-for-size graft in adult living donor liver transplantation. *Am J Transplant* 2008; **8**: 847-853 [PMID: 18261170 DOI: 10.1111/j.1600-6143.2007.02144.x]

11 **Court FG**, Wemyss-Holden SA, Morrison CP, Teague BD, Laws PE, Kew J, Dennison AR, Maddern GJ. Segmental nature of the porcine liver and its potential as a model for experimental partial hepatectomy. *Br J Surg* 2003; **90**: 440-444 [PMID: 12673745 DOI: 10.1002/bjs.4053]

12 **Itasaka H**, Suehiro T, Wakiyama S, Yanaga K, Shimada M, Sugimachi K. Significance of hyaluronic acid for evaluation of hepatic endothelial cell damage after cold preservation/reperfusion. *J Surg Res* 1995; **59**: 589-595 [PMID: 7475005 DOI: 10.1006/jsre.1995.1209]

13 **Eriksson S**, Fraser JR, Laurent TC, Pertoft H, Smedsrød B. Endothelial cells are a site of uptake and degradation of

hyaluronic acid in the liver. *Exp Cell Res* 1983; **144**: 223-228 [PMID: 6840207 DOI: 10.1016/0014-4827(83)90458-5]
14 **Kahn D**, Stadler J, Terblanche J, van Hoorn-Hickman R. Thymidine kinase: an inexpensive index of liver regeneration in a large animal model. *Gastroenterology* 1980; **79**: 907-911 [PMID: 7419015]
15 **Iwanaga S**, Morita T, Harada T, Nakamura S, Niwa M, Takada K, Kimura T, Sakakibara S. Chromogenic substrates for horseshoe crab clotting enzyme. Its application for the assay of bacterial endotoxins. *Haemostasis* 1978; **7**: 183-188 [PMID: 658779]
16 **Ludwig W**. Nucleic acid techniques in bacterial systematics and identification. *Int J Food Microbiol* 2007; **120**: 225-236 [PMID: 17961780 DOI: 10.1016/j.ijfoodmicro.2007.06.023]
17 **Amann RI**, Ludwig W, Schleifer KH. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* 1995; **59**: 143-169 [PMID: 7535888]
18 **Oliver RH**, Sutton PM. The effects of partial hepatectomy on portal pressure in rats. *Br J Surg* 1966; **53**: 138-141 [PMID: 4952331 DOI: 10.1002/bjs.1800530213]
19 **Gruttadauria S**, Mandala' L, Miraglia R, Caruso S, Minervini MI, Biondo D, Volpes R, Vizzini G, Marsh JW, Luca A, Marcos A, Gridelli B. Successful treatment of small-for-size syndrome in adult-to-adult living-related liver transplantation: single center series. *Clin Transplant* 2007; **21**: 761-766 [PMID: 17988271]
20 **Shimamura T**, Taniguchi M, Jin MB, Suzuki T, Matsushita M, Furukawa H, Todo S. Excessive portal venous inflow as a cause of allograft dysfunction in small-for-size living donor liver transplantation. *Transplant Proc* 2001; **33**: 1331 [PMID: 11267312 DOI: 10.1016/S0041-1345(00)02496-9]
21 **Ueno S**, Kobayashi Y, Kurita K, Tanabe G, Aikou T. Effect of prior portosystemic shunt on early hepatic hemodynamics and sinusoids following 84% hepatectomy in dogs. *Res Exp Med* (Berl) 1995; **195**: 1-8 [PMID: 7784699 DOI: 10.1007/BF02576768]
22 **Campos BD**, Botha JF. Strategies to optimize donor safety with smaller grafts for adult-to-adult living donor liver transplantation. *Curr Opin Organ Transplant* 2012; **17**: 230-234 [PMID: 22569511]
23 **Troisi R**, Cammu G, Militerno G, De Baerdemaeker L, Decruyenaere J, Hoste E, Smeets P, Colle I, Van Vlierberghe H, Petrovic M, Voet D, Mortier E, Hesse UJ, de Hemptinne B. Modulation of portal graft inflow: a necessity in adult living-donor liver transplantation? *Ann Surg* 2003; **237**: 429-436 [PMID: 12616129 DOI: 10.1097/01.SLA.0000055277.78876.B7]
24 **Chan SC**, Fan ST, Lo CM, Liu CL. Effect of side and size of graft on surgical outcomes of adult-to-adult live donor liver transplantation. *Liver Transpl* 2007; **13**: 91-98 [PMID: 17192891 DOI: 10.1002/lt.20987]
25 **Hessheimer AJ**, Fondevila C, Taurá P, Muñoz J, Sánchez O, Fuster J, Rimola A, García-Valdecasas JC. Decompression of the portal bed and twice-baseline portal inflow are necessary for the functional recovery of a "small-for-size" graft. *Ann Surg* 2011; **253**: 1201-1210 [PMID: 21587116 DOI: 10.1097/SLA.0b013e3181ffb2d7]
26 **Schindl MJ**, Redhead DN, Fearon KC, Garden OJ, Wigmore SJ. The value of residual liver volume as a predictor of hepatic dysfunction and infection after major liver resection. *Gut* 2005; **54**: 289-296 [PMID: 15647196 DOI: 10.1136/gut.2004.046524]
27 **Ito T**, Kiuchi T, Yamamoto H, Oike F, Ogura Y, Fujimoto Y, Hirohashi K, Tanaka AK. Changes in portal venous pressure in the early phase after living donor liver transplantation: pathogenesis and clinical implications. *Transplantation* 2003; **75**: 1313-1317 [PMID: 12717222 DOI: 10.1097/01.TP.0000063707.90525.10]
28 **Yagi S**, Iida T, Hori T, Taniguchi K, Yamamoto C, Yamagiwa K, Uemoto S. Optimal portal venous circulation for liver graft function after living-donor liver transplantation. *Transplantation* 2006; **81**: 373-378 [PMID: 16477223 DOI: 10.1097/01.tp.0000198122.15235.a7]
29 **Asakura T**, Ohkohchi N, Orii T, Koyamada N, Tsukamoto S, Sato M, Enomoto Y, Usuda M, Satomi S. Portal vein pressure is the key for successful liver transplantation of an extremely small graft in the pig model. *Transpl Int* 2003; **16**: 376-382 [PMID: 12819867 DOI: 10.1111/j.1432-2277.2003.tb00317.x]
30 **Botha JF**, Campos BD, Johanning J, Mercer D, Grant W, Langnas A. Endovascular closure of a hemiportocaval shunt after small-for-size adult-to-adult left lobe living donor liver transplantation. *Liver Transpl* 2009; **15**: 1671-1675 [PMID: 19938118 DOI: 10.1002/lt.21944]
31 **Medzhitov R**, Janeway C. Innate immunity. *N Engl J Med* 2000; **343**: 338-344 [PMID: 10922424 DOI: 10.1056/NEJM200008033430506]
32 **Yagi S**, Iida T, Hori T, Taniguchi K, Nagahama M, Isaji S, Uemoto S. Effect of portal haemodynamics on liver graft and intestinal mucosa after small-for-size liver transplantation in swine. *Eur Surg Res* 2012; **48**: 163-170 [PMID: 22653087 DOI: 10.1159/000338622]

P- Reviewer: Riehle KJ **S- Editor**: Gou SX **L- Editor**: A
E- Editor: Wang CH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.1030

World J Gastroenterol 2014 January 28; 20(4): 1030-1037
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Novel esophageal squamous cell carcinoma bone metastatic clone isolated by scintigraphy, X ray and micro PET/CT

Bi-Zeng Zhao, Jie Cao, Jin-Chen Shao, Yan-Bing Sun, Li-Min Fan, Chun-Yu Wu, Sheng Liang, Bao-Feng Guo, Guang Yang, Wen-Hui Xie, Qing-Cheng Yang, Shun-Fang Yang

Bi-Zeng Zhao, Qing-Cheng Yang, Department of Orthopedics, Shanghai Sixth Hospital of Shanghai Jiaotong University, Shanghai 200233, China
Jie Cao, Wen-Hui Xie, Shun-Fang Yang, Department of Nuclear Medicine, Shanghai Chest Hospital of Shanghai Jiaotong University, Shanghai 200030, China
Jin-Chen Shao, Department of Pathology, Shanghai Chest Hospital of Shanghai Jiaotong University, Shanghai 200030, China
Yan-Bing Sun, Department of Radiology, Shanghai Chest Hospital of Shanghai Jiaotong University, Shanghai 200030, China
Li-Min Fan, Department of Thoracic Surgery, Shanghai Chest Hospital of Shanghai Jiaotong University, Shanghai 200030, China
Chun-Yu Wu, Bao-Feng Guo, Department of Breast Surgery, Shanghai Long Hua Hospital of Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China
Sheng Liang, Micro PET/CT Center, Department of Nuclear Medicine, Shanghai RuiJin Hospital of Shanghai Jiaotong University, Shanghai 200025, China
Guang Yang, Kyocera Communication Systems (Shanghai) Co.,Ltd, Shanghai 201203, China
Author contributions: Zhao BZ and Fan LM were responsible for animal experiments and collection of human specimens; Cao J and Xie WH contributed to the tail vein injection of radionuclide and pinhole bone scintigraphy; Yang QC participated in experimental design; Shao JC contributed to pathological diagnosis; Sun YB contributed to human conventional radiography used in the mice; Wu CY and Guo BF contributed to statistical analysis and real-time polymerase chain reaction; Liang S contributed to micro positron emission/computed tomography; Yang G contributed to images processing; Yang SF designed the study and wrote the manuscript; all authors read and approved the final version.
Supported by Shanghai Science and Technology fundamental research Grant 08140902202 and 09140901500 (to Yang SF); and National Natural Science Foundation of China Grant 30973017 (to Yang QC)
Correspondence to: Yang Shun-Fang, Associate Professor, Department of Nuclear Medicine, Shanghai Chest Hospital, Shanghai Jiaotong University, Shanghai 200030, China. yzyg@sh163.net
Telephone: +86-21-62821990 Fax: +86-21-62801109;
Received: April 24, 2013 Revised: September 21, 2013
Accepted: September 29, 2013
Published online: January 28, 2014

Abstract

AIM: To establish a Chinese esophageal squamous cell carcinoma (ESCC) cell line with high bone metastasis potency using ^{99m}Tc-methylene diphosphonate (^{99m}Tc-MDP) micro-pinhole scintigraphy, X ray and micro-positron emission tomography/computed tomography (PET/CT) for exploring the mechanism of occurrence and development in esophageal cancer.

METHODS: The cells came from a BALB/c nu/nu immunodeficient mouse, and oncogenic tumor tissue was from a surgical specimen from a 61-year-old male patient with ESCC. The cell growth curve was mapped and analysis of chromosome karyotype was performed. Approximately 1 × 10^6 oncogenic cells were injected into the left cardiac ventricle of immunodeficient mice. The bone metastatic lesions of tumor-bearing mice were detected by ^{99m}Tc-MDP scintigraphy, micro-PET/CT and X-ray, and were resected from the mice under deep anesthesia. The bone metastatic cells in the lesions were used for culture and for repeated intracardiac inoculation. This *in vivo*/*in vitro* experimental metastasis study was repeated for four cycles. All of the suspicious bone sites were confirmed by pathology. Real-time polymerase chain reaction was used to compare the gene expression in the parental cells and in the bone metastatic clone.

RESULTS: The surgical specimen was implanted subcutaneously in immunodeficient mice and the tumorigenesis rate was 100%. First-passage oncogenic cells were named CEK-Sq-1. The chromosome karyotype analysis of the cell line was hypotriploid. The bone

metastasis rate went from 20% with the first-passage oncogenic cells *via* intracardiac inoculation to 90% after four cycles. The established bone metastasis clone named CEK-Sq-1BM had a high potential to metastasize in bone, including mandible, humerus, thoracic and lumbar vertebrae, scapula and femur. The bone metastasis lesions were successfully detected by micro-pinhole bone scintigraphy, micro-PET/CT, and X-ray. The sensitivity, specificity and accuracy of the micro-pinhole scintigraphy, X-ray, and micro-PET/CT imaging examinations were: 89.66%/32%/80%, 88.2%/100%/89.2%, and 88.75%/77.5%/87.5%, respectively. Some gene expression difference was found between parental and bone metastasis cells.

CONCLUSION: This newly established Chinese ESCC cell line and animal model may provide a useful tool for the study of the pathogenesis and development of esophageal carcinoma.

Key words: Esophageal squamous cell carcinoma; Cell line; Bone metastasis; Molecular imaging; Real-time polymerase chain reaction

Core tip: We established a novel esophageal squamous cell carcinoma cell line from a surgically resected human specimen and its clone with mixed bone metastasis potency by molecular imaging including conventional radiography, bone scintigraphy, and micro-positron emission tomography/computed tomography after intracardiac inoculation of the cells into nude mice. The process was repeated *in vivo* and *in vitro* for four cycles to obtain a bone metastasis clone CEK-Sq-1BM. Some gene expression difference was compared with the primary cells and their clone by real-time reverse transcriptase polymerase chain reaction. This work may be helpful for research in bone metastases.

Zhao BZ, Cao J, Shao JC, Sun YB, Fan LM, Wu CY, Liang S, Guo BF, Yang G, Xie WH, Yang QC, Yang SF. Novel esophageal squamous cell carcinoma bone metastatic clone isolated by scintigraphy, X ray and micro PET/CT. *World J Gastroenterol* 2014; 20(4): 1030-1037 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/1030.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.1030

INTRODUCTION

Esophageal cancer (EC) is among the top six leading causes of death from cancer; it exhibits a strikingly uneven geographical distribution, resulting in focal endemic high-incidence areas in several countries[1], including Northern China, Northern Iran and South Africa. Considerable advances in diagnosis, surgical techniques and chemoradiotherapy have been made for EC. Nevertheless, EC remains one of the most lethal cancers and most patients die from recurrence or metastasis, with a 5-year survival rate as low as 16% in the United States in 1996-2004, and it was ranked fifth among the five leading causes of cancer mortality in male patients aged 40-79 years in 2008[2,3]. In China, the situation is even worse[4]. The most common metastatic sites of EC are lung, liver and bone. The median survival time is < 1 year for advanced cases. To improve survival for EC patients, a better understanding of the cellular and molecular carcinogenesis of EC, and especially of the mechanism of metastasis, is needed. Well-characterized human EC cell lines and animal models for metastasis are important research resources for studying cancer cell biology, as well as for developing new strategies against EC. Although there are many EC cell lines[5-9], a bone metastasis model is lacking. To enrich the bank of cell lines and animal models of EC, a new human EC cell line (CEK-Sq-1) and its clone with a high bone-seeking tendency were established. Their morphological and biological characteristics and tumorigenicity *in vivo* were described in this experimental study, which may provide a useful model for *in vitro* and *in vivo* cellular and molecular research.

MATERIALS AND METHODS

With the patient's consent, an esophageal squamous cell carcinoma (ESCC) tumor specimen was surgically removed from a 61-year-old Chinese man. The tumor was diagnosed pathologically as T2N1M0 after surgery. Lymph node metastasis occurred 6 mo later and bone metastasis 1 year after the operation. Fresh tumor tissue specimens were cut into small pieces of about 1 mm^3 and soaked with 40000 U/mL penicillin and 1000 μg/mL streptomycin (Northern China Pharmaceutical, Shijiazhuang, China) for about 5 min.

Xenograft and cell culture

The specimens were implanted subcutaneously in three immunodeficient mice and the tumorigenesis rate was 100% after 4 wk. The xenograft tumor was excised after the mouse was deeply anesthetized and then sacrificed. The tumor was minced for cell culture in 25-cm^2 culture flasks with 6 mL RPMI 1640 medium (Gibco, Carlsbad, CA, United States) containing 10% fetal bovine serum (Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (Northern China Pharmaceutical). The cells were cultured at 37 ℃ in an atmosphere with 5% CO_2. The first-passage oncogenic cells were cultured after growing for 7 d. The cells were named CEK-Sq-1.

Chromosome analysis

The CEK-Sq-1 cells at the 15th passage at the stage of proliferation were selected and treated with colchicine for 4-6 h. The cell suspension was collected and Giemsa-stained after hypotonic treatment. We selected cells with well-dispersed chromosomes in metaphase. The chromosome distribution and karyotype features were investigated by analyzing 40 and 15 metaphases, respectively.

Growth characteristics of the cell line

The CEK-Sq-1 cells of the 7th, 24th and 40th passages were studied to estimate the population-doubling time. A suspension of 1×10^5 cells was plated into 25-cm^2 culture flasks. The number of viable cells from three culture flasks per passage was measured with a Neubauer hemocytometer every 24 h for 8 d by trypan blue staining. The growth curve was plotted and the population doubling time of the CEK-Sq-1 cell line was calculated during the exponential growth phase of the cells and using online algorithm software provided at http://www.doubling-time.com.

Establishment of the human experimental bone metastasis clone

The experimental animal study protocols were approved by the Shanghai Laboratory Animal Science Administration Commission of Shanghai Municipality. All BALB/c nu/nu nude mice (Shanghai Cancer Institute of Shanghai Jiaotong University, Shanghai, China) were maintained in a specific pathogen-free environment. Cells in the fifth passage were harvested from cell culture flasks and resuspended at 1×10^7/mL in phosphate buffered solution. Following this, 0.1 mL of the cell suspension was injected into the left cardiac ventricles of 8-wk-old male mice using 29 G needles. The mice were anesthetized as previously described [10].

To begin with, the bone metastasis rate was 20% (2/10) for the fifth passage cells. The bone metastasis tumor clones were dissected and the cells cultured. On the second cycle of inoculation, about 40% (4/10) of the transplanted mice had bone metastasis. Starting with the fourth cycle, 90% of the transplanted mice had observable bone metastasis. Most of the bone metastasis sites were in the mandible, spine and four limbs; the other bone metastasis sites were the skull and ribs. The fourth cycle of the bone-seeking cells was named CEK-Sq-1BM.

Bone metastatic imaging

BALB/c mice were deeply anesthetized with an intraperitoneal injection of 75-100 mg/kg thiopental. Bone metastasis was evaluated by *in vivo* imaging weekly micropinhole bone scitigraphy with ^{99m}Tc-methylene diphosphonate (^{99m}Tc-MDP; Shanghai Syncor Pharmaceutical, Shanghai, China) starting 4 wk after inoculation. Static planar images of the entire skeleton were acquired 5-6 h after tail vein injection of 111 MBq (3 mCi), or 0.1 mL ^{99m}Tc-MDP on a GE Hawkeye 4 Infinia Functional Imaging Scanner (GE Medical Systems, Waukesha, WI, United States) with a pinhole collimator. The pinhole insert had been designed and built for obtaining ultra-high resolution images, with an aperture diameter of 1 mm. Radiography images were used as controls. Conventional radiographs were obtained with a Philips Optimus Bucky Diagnost TS X-ray System (Philips Healthcare, Eindhoven, The Netherlands). Bone metastases were determined using the radiograph tube voltage fixed at 40 kVp, the current at 2 mA, and the exposure time at 3 s[10].

Micro PET/CT imaging

A high resolution, animal positron emission tomography/computed tomography (PET/CT) scanner (Inveon micro PET/CT, Siemens Preclinical Solution, Knoxville, TN, United States) was used to image the BALB/c nu-nu nude mice. For each imaging session, awake mice were injected with 0.15-0.2 mCi of ^{18}F-FLT, given tail vein injection. Each mouse was anesthetized with 5% isofluorane in an induction chamber. Afterwards, the mouse was placed in the scanner bed in the prone position with the long axis of the heart parallel and within the FOV of the scanner. During image acquisition, the mice were anesthetized with 1%-2% isofluorane gas delivered through a custom face mask.

All micro-PET images were reconstructed with the standard ordered-subset expectation maximization (OSEM) method. Reconstructed images were displayed in transverse, coronal and sagittal planes. A total of 512 sequential tomographic slices were displayed over the imaging object of interest with each slice measuring 0.11 mm in thickness, and assessed visually using transaxial, sagittal, and coronal displays. CT images were used for both attenuation correction of emission data and image fusion.

Histological examination

Every tumor-bearing nude mouse was sacrificed with deep anesthesia 45 d after inoculation and cut into eight skeleton sites (mandible, spine and both scapula, humerus, femur) for histological diagnosis. Bone metastasis clones were observed by radionuclide scintigraphy and radiography, and the bony tissue was dissected and formalin soaked, decalcified, embedded in paraffin, cut in thin sections, and stained with hematoxylin and eosin (H&E). Each of the slides was examined independently by two pathology specialists.

Real-time PCR

Total RNA was extracted from parental CEK-Sq-1 and CEK-Sq-1BM cells with TRIzol reagent (Gibco). Quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was used to validate gene expressions of the bone metastasis cells. Reverse transcription was performed using the Reverse Transcriptase Kit (Promega, San Luis Obispo, CA, United States) following the manufacturer's instructions. We performed a set of real-time PCR assays with the approximation method using the ΔΔCT method. In the ΔΔCT method, GAPDH served as the endogenous control and the examined genes were *CDH1* (E-cadherin), *SERPINA1*, *SERPINE2*, *FN1*, *POU5F1* (OCT_4) and *AR*. The quantitative real-time PCR was performed in triplicate using SYBR Green Mastermix (TaKaRa, Kyoto, Japan) on the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, United States). PCR conditions were 95 ℃ for 15 s, 45 cycles of 95 ℃ for 20 s, 60 ℃ for 20 s and 72 ℃ for 20 s. The PCR primer sequences are shown in Table 1.

Table 1 Primers for real-time polymerase chain reaction

Gene name	Forward primer	Reverse primer
POU5F1	GTAGGTTCTTGAATCCCGAATG	TCTGCTTTGCATATCTCCTGAA
FN1	CTGCTGGGACTTCCTATGTGGT	GGTTTCCTCGATTATCCTTCTTG
CDH1	CTTCTGCTGACCTGTCTGATG	GTCACACACGCTGACCTCTAAG
SERPINE2	TCAGCACCAAGACCATAGACAG	CGGATGAAAAACAGAAAAGGTC
SRPINA1	AACGATTACGTGGAGAAGGGTA	GGTAAATGTAAGCTGGCAGACC
AR	ATTGAGCCAGGTGTAGTGTGTG	GGAGTTGACATTGGTGAAGGAT

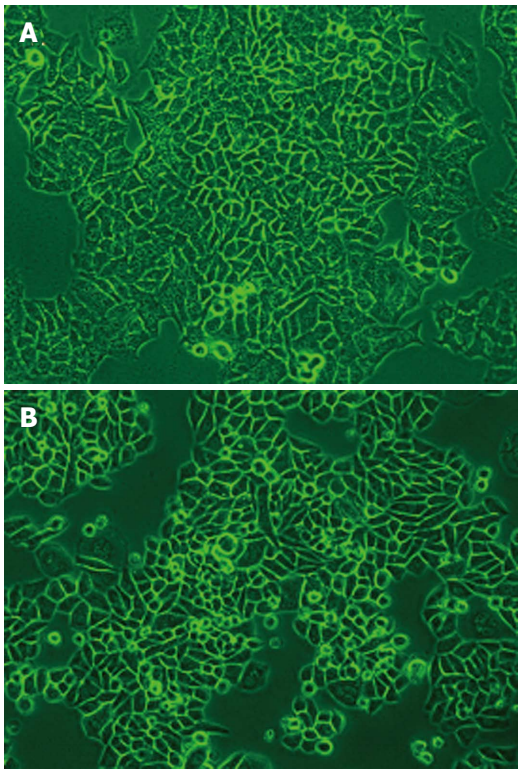


Figure 1 Morphology of human esophageal squamous cell carcinoma cell line CEK-Sq-1. A: First passage oncogenic cells (× 100); B: After four cycles, the bone-seeking subline CEK-Sq-1BM was harvested.

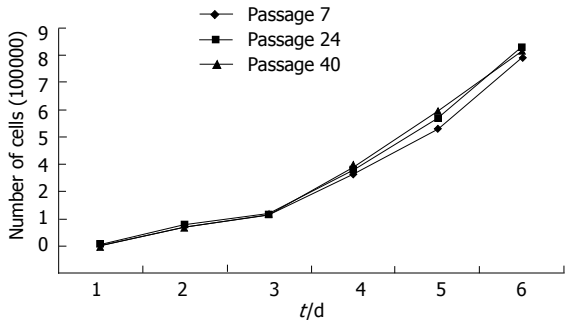


Figure 2 Growth curve of the CEK-Sq-1 cell line at passages 7, 24 and 40.

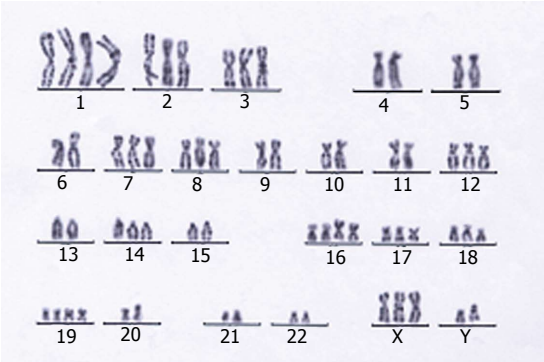


Figure 3 Chromosomal imbalances of CEK-Sq-1 shows the DNA over-representation on chromosomes 1-3, 7, 8, 12, 14, 16–19, X and Y.

RESULTS

Morphologic characterization

The CEK-Sq-1 cells from the xenograft tumor were adherent and tended to form a monolayer with epithelial features. Many morphologically distinct populations were found, such as small rounded, polygonal and spindle cells. Such heterogeneous cell types were observed from the first-passage oncogenic cells in culture (Figure 1A). The morphology of the cells remained unchanged even after 40 passages. After four cycles *in vivo*/*in vitro*, the bone-seeking clone CEK-Sq-1BM was harvested (Figure 1B).

Growth curve and doubling time

Figure 2 shows the growth curve and population doubling time of the established ESCC cell line; the population doubling was calculated from the growth curve. The analyzed cells were from the 7th, 24th and 40th passages. The population doubling time of the ESCC cells was about 46 h.

Karyotype

Karyotypic study revealed both numerical and structural abnormalities in this ESCC cell line. The modal number of chromosomes ranged from 58 to 68, with a median of 64. Most of the analyzable metaphases were hypotriploid (Figure 3).

Bone metastasis images

The bone metastasis lesions were detected by ^{99m}Tc-MDP micro-pinhole bone scintigraphy, X-ray, and micro-PET/CT. CEK-Sq-1BM was obtained after four cycles of the *in vivo*/*in vitro* repeat. The suspicious bone metastasis sites were scanned weekly under anesthesia 4 wk after inoculation of CEK-Sq-1 cells. The bone metastasis images of CEK-Sq-1BM mice were acquired by X-rays, micro-pinhole bone scintigraphy and micro-PET/CT (Figures 4 and 5). The whole-body X-ray image showed osteo-

Table 2 Sensitivity, specificity and accuracy with different techniques for pathology using χ^2 test

	Micro-pinhole BS (n = 7)		X- ray (n = 7)		Micro-PET/CT (n = 7)	
	+	-	+	-	+	-
Histology +	18	2	6	13	16	3
-	4	32	0	37	4	33

PET/CT: Positron emission tomography/computed tomography.

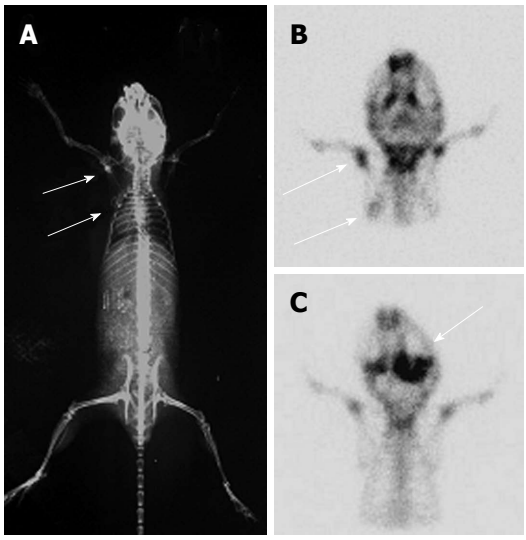


Figure 4 Whole-body X-ray and micro-pinhole bone scintigraphy images of experimental bone metastasis in mice. A: X-ray image, upper arrow indicates the hot spot of the left humeral osteoblastic metastatic lesion, and the lower arrow indicates the cold spot of the left scapular osteolytic metastatic lesion and circle around the osteogenic reaction; B: Micro-pinhole bone scintigraphy images of the same mouse in the POST position. The osteoblastic metastatic lesion (upper arrow) showed greater accumulation of ^{99m}Tc-methylene diphosphonate (^{99}mTc-MDP), and the osteolytic lesion (lower arrow) combined with osteogenic reaction showed less; C: The arrow indicates a mandibular lesion in another mouse with accumulation of ^{99}mTc-MDP.

blastic metastasis concurrent with osteolytic in a CEK-Sq-1 BM mouse at 6-8 wk (Figure 4A). The difference of osteoblastic and osteolytic metastasis presented in the same mouse by micro-pinhole bone scintigraphy (Figure 4B). The mandibular lesions of another model mouse accumulated ^{99m}Tc-MDP (Figure 4C). All of the suspicious bone metastasis sites were evaluated by histological examination after the mice were sacrificed, and the sensitivity, specificity and accuracy of micro-pinhole bone scintigraphy, X-ray, and micro-PET/CT imaging examinations were: 89.66%/32%/80%, 88.2%/100%/89.2%, and 88.75%/77.5%/87.5%, respectively (Table 2).

Histological examination

Figure 6 shows the histological examination of mandible (A) and lumbar vertebrae (B), confirming the bone metastasis sites (stained with H&E, magnification × 100 and 200, respectively).

Real-time PCR analysis of CEK-Sq-1 and CEK-Sq-1BM

Ratios of CEK-Sq-1BM compared to CEK-Sq-1. Can-

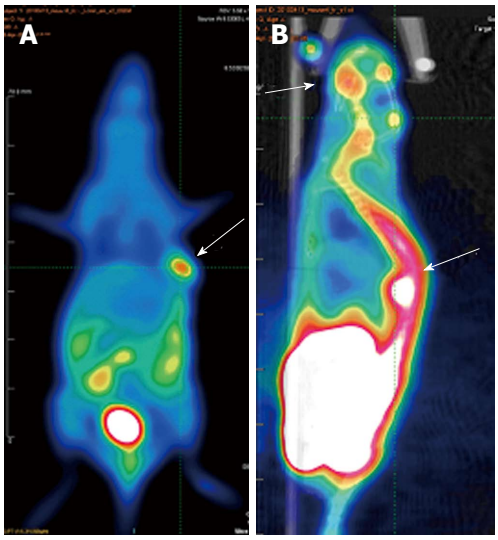


Figure 5 ^{18}F-FLT micro-PET/CT images of CEK-Sq-1MB mouse. A: Arrow indicates ^{18}F-FLT uptake positivity in the cells (2 × 10^6) injected subcutaneously into the left lateral subcutis of the mouse for 3 wk; B: Arrows indicate the mandibular and thoracic vertebral metastatic lesions of the cells after intracardiac injection in mice for 4 wk.

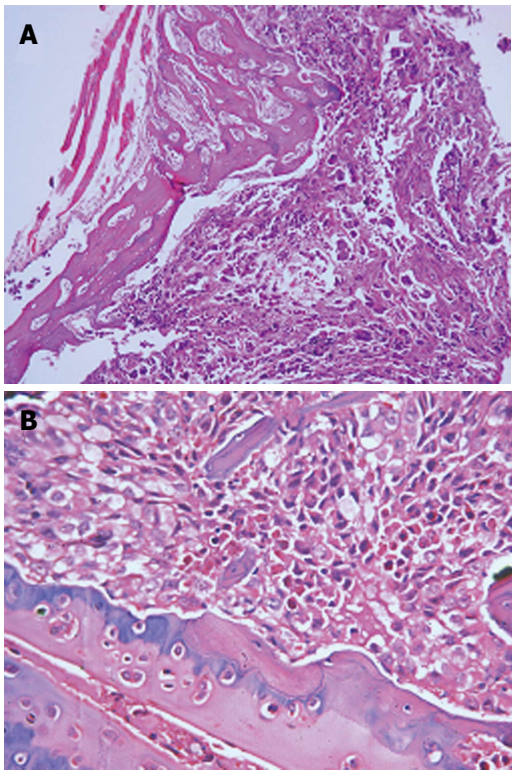


Figure 6 Histological features of the the mandibular (A) (hematoxylin and eosin, × 100) and lumbar vertebral (B) (hematoxylin and eosin, × 200) metastatic lesions.

cer invasion and metastasis are complex multistep processes[11]. Cancer cells invasion into blood vessels initiates metastasis, and the vessels also provide the routes for systemic spread of cancer cells[12]. Low apoptosis leads to clonal expansion and continuous selection of progressively more malignant cell populations[13]. Cell-cell adhesion plays an important role in metastasis too[14]. Thus, to

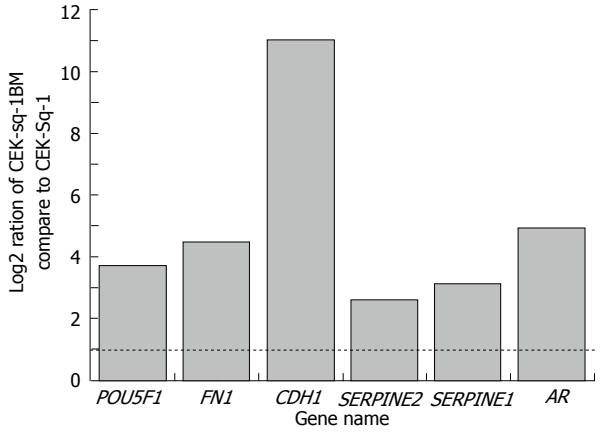


Figure 7 Expression changes of genes determined in CEK-Sq-1BM cells compared with CEK-Sq-1 cells.

examine that CEK-Sq-1BM had metastasis capacity compared with that of the CEK-Sq-1, we performed real-time PCR to detect changes in gene expression. Genes associated with cell adhesion molecules, such as *CDH1*, *SERPINA1*, *SERPINE2*, *FN1*, *POU5F1* and *AR* were examined.

As shown in Figure 7, the expression of CDH1, AR, FN1 and POU5F1 was increased in CEK-Sq-1BM compared to that in CEK-Sq-1 cells, while the expression of SERPINE2 and SERPINA1 was moderately increased. We selected these genes that were overexpressed in cancer metastasis by real-time PCR analysis, and confirmed their high expression in CEK-Sq-1BM cells. This indicated that the clone did indeed have metastatic capacity compared to its parental CEK-Sq-1 cells.

DISCUSSION

EC is one of the least studied and deadliest cancers worldwide; the treatment of EC, especially advanced stage, remains challenging. Therefore, cell lines and human xenograft models that accurately mimic human EC are needed to elucidate the biological characteristics of these tumors. However, the available and useful cell lines and animal models of ESCC remain limited, especially for metastatic models.

The first melanoma bone metastasis animal model was established early in 1988[15]. Breast cancer[16], prostate cancer[17], and small cell lung cancer bone metastasis models[18] were then successfully established. However, to the best of our knowledge, no experimental EC bone metastasis models have been reported so far. One of the important reasons may be the lack of a highly potent bone metastasis cell line. Second, the modeling methods are labor intensive, involving anesthesia, cancer cell injection into the left cardiac ventricle of mice, and then resuscitation. Third, ^{99m}Tc-MDP micro-pinhole bone scintigraphy and micro-SPECT/CT, which can detect osteoblastic metastases with great sensitivity, have only recently been developed. Fourth, ^{18}F-fluoride micro-PET/CT scans mainly detect osteolytic metastases, which is also the limitation of X-rays, which is the most commonly used method to search for bone metastasis, requiring about 30%-75% reduction in bone density to visualize a metastasis[19].

During the lifespan of a tumor-bearing mouse, which is generally 30-60 d, the metastasis sites may not be observed by X-rays because of limited bone density reduction. For detecting bone metastasis with bone scintigraphy, at least a 5%-10% change in the ratio of lesion to normal bone is required to detect an abnormality. It has been estimated that bone scintigraphy can detect malignant bone lesions 2-18 mo earlier than plain radiography, and bone scintigraphy sensitivities in detecting bone metastases vary between 62% and 100% with specificity of 78%-100%. This is because the alteration in osteoblastic action and/or blood supply occurs at an earlier stage than the difference in bone density needed to produce a radiographic change[20,21]. For bone metastasis examination, bone scintigraphy could have a similar sensitivity and specificity to PET/CT, but with a relative economic advantage leading to more common use in clinical and experimental research[22].

We established an experimental lung adenocarcinoma bone metastasis cell line and mouse model in 2009 with a gamma camera using ^{99m}Tc-MDP micro-pinhole bone scintigraphy, which was the first and is still the only lung adenocarcinoma model[10]. The same technique was used to establish this new EC cell line with high bone metastasis potency. All of the suspicious bone metastasis sites were confirmed by pathological examination, with a sensitivity of 89.66% and a specificity of 88.2%, which demonstrates the advantage of micro-pinhole bone scintigraphy over X-rays.

The formation of bony metastases is a multi-gene, multi-step synergistic process that includes intravasation of tumor cells from the primary site into the bloodstream, survival while in the circulation, adhesion to endothelial cells within the target organ, and extravasation from the bloodstream into the surrounding tissue[23] or marrow cavity. Cell-cell adhesion molecules SERPINE2 and SERPINA1 have been proven to play important roles in the increased metastasis and invasion potency of lung adenocarcinoma, pancreatic and colorectal tumors[10,24,25]. FN1, a cellular adhesion factor, is associated with tumor metastasis and forms a highly interactive network in ESCC[26]. In renal cancer[27], melanoma[28] and non-small cell lung cancer[29], FN1 is required for cell invasion and metastasis. CDH1 is a 120-kDa transmembrane glycoprotein belonging to the Super family of Ca^2-dependent cell adhesion molecules and its role remains controversial in malignant progression[30,31]. In addition, E-cadherin is one of the cell surface markers of human embryonic stem cells (hESCs)[32]. The bone metastasis and primary tissue sections of prostate cancer were stained by immunohistochemistry using specific antibody to CDH1, which showed CDH1 overexpression in bone metastasis[33]. CDH1 upregulation may be related to osteolytic or

mixed bone metastasis. POU5F1, a member of the POU (Pit-oct-unc) family of transcriptional factors, is essential for maintenance of the stem character in hESCs. The expression of POU5F1 has been proven to increase the risk of metastasis and markedly enhance resistance to cytotoxic agents[34]. These hESC markers (E-cadherin and OCT4) may promote the initiation, progression, and differentiation of human cancers. Androgen receptors (ARs) are probably of importance during all phases of prostate cancer growth and assessment of breast cancer prognosis, but their role in bone metastases is largely unexplored[35,36].

In conclusion, we established a new ESCC cell line, CEK-Sq-1, from ESCC tissue, which had high bone metastatic potency. To the best of our knowledge, no investigations have been reported of cell lines and bone metastasis animal models with EC tumors. This newly established cell line and animal model may provide a useful tool for the study of the pathogenesis of EC.

ACKNOWLEDGMENTS

We thank Chengmo Zhu, MD; Jixiao Ma, MD; Meiping Shi, MD; Jiuxian Feng, MD; Ning Zheng, PhD; Yifeng Jiang, MD; Zhichun Zheng, MD for scientific suggestions and technical help. We thank the technicians of radiology department at Shanghai Chest Hospital of Shanghai Jiaotong University for their supports.

COMMENTS

Background

Esophageal cancer (EC) is among the top six leading causes of death from recurrence or metastasis, with a 5-year survival rate as low as 16% in the United States. In China, the situation is even worse, especially in the north. The most common metastatic sites of EC are lung, liver and bone. To improve survival for EC patients, a better understanding of the cellular and molecular carcinogenesis, especially of the mechanism of metastasis, is needed, but a bone metastasis model is lacking. The authors established a new human EC cell line (CEK-Sq-1) and its clone with a high bone-seeking tendency. Their morphological and biological characteristics and tumorigenicity *in vivo* were described in this experimental study. The results may provide a useful model for *in vitro* and *in vivo* cellular and molecular research.

Research frontiers

The cells came from oncogenic tissue in a BALB/c nu/nu immunodeficient mouse and from a surgical specimen from a 61-year-old male Chinese patient with esophageal squamous cell carcinoma (ESCC). The oncogenic cells were injected into the left cardiac ventricle of immunodeficient mice. The bone metastatic lesions of tumor-bearing mice were detected by ^{99m}Tc-methylene diphosphonate (^{99m}Tc-MDP) scintigraphy, micro-positron emission tomography/computed tomography and X-rays, and were resected from the mice under deep anesthesia. The bone metastatic cells in the lesions were used for culture and repeated intracardiac inoculation. This *in vivo*/*in vitro* study was repeated four times. All of the suspicious bone sites were confirmed by pathology. Real-time polymerase chain reaction was used to compare the gene expression in the parental cells (CEK-Sq-1) and in the bone metastatic clone (CEK-Sq-1BM).

Innovations and breakthroughs

A novel ESCC cell line (CEK-Sq-1) and its bone metastasis clone (CEK-Sq-1BM) were established from a Chinese patient by molecular imaging. Some differences in gene expression and the characteristics of the bone metastatic lesions were revealed, which could form the basis for further research into ESCC.

Applications

This study established a new research method that could be used for studies on ESCC, including tumor cells, animal models and tumor markers.

Peer review

The authors first showed the morphology of the newly established ESSC cell line and molecular imaging of its bone-seeking clone. Most importantly, the authors showed the karyotype of this cell line, identified the type of bone metastasis and hypothesized markers, which is especially important.

REFERENCES

1 **Jemal A**, Center MM, DeSantis C, Ward EM. Global patterns of cancer incidence and mortality rates and trends. *Cancer Epidemiol Biomarkers Prev* 2010; **19**: 1893-1907 [PMID: 20647400 DOI: 10.1158/1055-9965.EPI-10-0437]
2 **Jemal A**, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. *CA Cancer J Clin* 2009; **59**: 225-249 [PMID: 19474385 DOI: 10.3322/caac.20006]
3 **Siegel R**, Naishadham D, Jemal A. Cancer statistics, 2012. *CA Cancer J Clin* 2012; **62**: 10-29 [PMID: 22237781 DOI: 10.3322/caac.20138]
4 **Yang L**, Parkin DM, Li L, Chen Y. Time trends in cancer mortality in China: 1987-1999. *Int J Cancer* 2003; **106**: 771-783 [PMID: 12866039 DOI: 10.1002/ijc.11300]
5 **Shimada Y**, Imamura M, Wagata T, Yamaguchi N, Tobe T. Characterization of 21 newly established esophageal cancer cell lines. *Cancer* 1992; **69**: 277-284 [PMID: 1728357]
6 **Zhang QB**, Gao YP, He JT, Zhang TT, Lin P, Zhang J, Wang XJ. Establishment of a novel human esophageal squamous cell carcinoma cell line (ESC-410) and its partial biological characterization. *Dis Esophagus* 2011; **24**: 120-126 [PMID: 20819098 DOI: 10.1111/j.1442-2050.2010.01106.x]
7 **Hu YC**, Lam KY, Law SY, Wan TS, Ma ES, Kwong YL, Chan LC, Wong J, Srivastava G. Establishment, characterization, karyotyping, and comparative genomic hybridization analysis of HKESC-2 and HKESC-3: two newly established human esophageal squamous cell carcinoma cell lines. *Cancer Genet Cytogenet* 2002; **135**: 120-127 [PMID: 12127396 DOI: 10.1016/S0165-4608(01)00580-5]
8 **Sarbia M**, Bösing N, Hildebrandt B, Koldovsky P, Gerharz CD, Gabbert HE. Characterization of two newly established cell lines derived from squamous cell carcinomas of the oesophagus. *Anticancer Res* 1997; **17**: 2185-2192 [PMID: 9216685]
9 **Mok CH**, Chew EC, Riches DJ, Lee JC, Huang DP, Hadgis C, Crofts TJ. Biological characteristics of a newly established human oesophageal carcinoma cell line. *Anticancer Res* 1987; **7**: 409-415 [PMID: 3631894]
10 **Yang S**, Dong Q, Yao M, Shi M, Ye J, Zhao L, Su J, Gu W, Xie W, Wang K, Du Y, Li Y, Huang Y. Establishment of an experimental human lung adenocarcinoma cell line SPC-A-1BM with high bone metastases potency by (99m)Tc-MDP bone scintigraphy. *Nucl Med Biol* 2009; **36**: 313-321 [PMID: 19324277 DOI: 10.1016/j.nucmedbio.2008.12.007]
11 **Liotta LA**, Wewer U, Rao NC, Schiffmann E, Stracke M, Guirguis R, Thorgeirsson U, Muschel R, Sobel M. Biochemical mechanisms of tumor invasion and metastases. *Prog Clin Biol Res* 1988; **256**: 3-16 [PMID: 2835781]
12 **O'Reilly MS**, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 1994; **79**: 315-328 [PMID: 7525077 DOI: 10.1016/0092-8674(94)90200-3]
13 **Reya T**, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001; **414**: 105-111 [PMID: 11689955 DOI: 10.1038/35102167]
14 **Mareel M**, Leroy A. Clinical, cellular, and molecular aspects of cancer invasion. *Physiol Rev* 2003; **83**: 337-376 [PMID: 12663862]
15 **Arguello F**, Baggs RB, Frantz CN. A murine model of experimental metastasis to bone and bone marrow. *Cancer Res* 1988; **48**: 6876-6881 [PMID: 3180096]
16 **Sasaki A**, Boyce BF, Story B, Wright KR, Chapman M, Boyce

R, Mundy GR, Yoneda T. Bisphosphonate risedronate reduces metastatic human breast cancer burden in bone in nude mice. *Cancer Res* 1995; **55**: 3551-3557 [PMID: 7627963]
17 **Rabbani SA**, Harakidas P, Bowlin T, Attardo G. Effect of nucleoside analogue BCH-4556 on prostate cancer growth and metastases in vitro and in vivo. *Cancer Res* 1998; **58**: 3461-3465 [PMID: 9699681]
18 **Miki T**, Yano S, Hanibuchi M, Kanematsu T, Muguruma H, Sone S. Parathyroid hormone-related protein (PTHrP) is responsible for production of bone metastasis, but not visceral metastasis, by human small cell lung cancer SBC-5 cells in natural killer cell-depleted SCID mice. *Int J Cancer* 2004; **108**: 511-515 [PMID: 14696114 DOI: 10.1002/ijc.11586]
19 **Even-Sapir E**. Imaging of malignant bone involvement by morphologic, scintigraphic, and hybrid modalities. *J Nucl Med* 2005; **46**: 1356-1367 [PMID: 16085595]
20 **Blake GM**, Park-Holohan SJ, Cook GJ, Fogelman I. Quantitative studies of bone with the use of 18F-fluoride and 99mTc-methylene diphosphonate. *Semin Nucl Med* 2001; **31**: 28-49 [PMID: 11200203 DOI: 10.1053/snuc.2001.18742]
21 **Rybak LD**, Rosenthal DI. Radiological imaging for the diagnosis of bone metastases. *Q J Nucl Med* 2001; **45**: 53-64 [PMID: 11456376]
22 **Virk MS**, Petrigliano FA, Liu NQ, Chatziioannou AF, Stout D, Kang CO, Dougall WC, Lieberman JR. Influence of simultaneous targeting of the bone morphogenetic protein pathway and RANK/RANKL axis in osteolytic prostate cancer lesion in bone. *Bone* 2009; **44**: 160-167 [PMID: 18929692 DOI: 10.1016/j.bone.2008.09.009]
23 **Chambers AF**, Groom AC, MacDonald IC. Dissemination and growth of cancer cells in metastatic sites. *Nat Rev Cancer* 2002; **2**: 563-572 [PMID: 12154349 DOI: 10.1038/nrc865]
24 **Buchholz M**, Biebl A, Neesse A, Wagner M, Iwamura T, Leder G, Adler G, Gress TM. SERPINE2 (protease nexin I) promotes extracellular matrix production and local invasion of pancreatic tumors in vivo. *Cancer Res* 2003; **63**: 4945-4951 [PMID: 12941819]
25 **Bergeron S**, Lemieux E, Durand V, Cagnol S, Carrier JC, Lussier JG, Boucher MJ, Rivard N. The serine protease inhibitor serpinE2 is a novel target of ERK signaling involved in human colorectal tumorigenesis. *Mol Cancer* 2010; **9**: 271 [PMID: 20942929 DOI: 10.1186/1476-4598-9-271]
26 **Wong FH**, Huang CY, Su LJ, Wu YC, Lin YS, Hsia JY, Tsai HT, Lee SA, Lin CH, Tzeng CH, Chen PM, Chen YJ, Liang SC, Lai JM, Yen CC. Combination of microarray profiling and protein-protein interaction databases delineates the minimal discriminators as a metastasis network for esophageal squamous cell carcinoma. *Int J Oncol* 2009; **34**: 117-128 [PMID: 19082484]
27 **Waalkes S**, Atschekzei F, Kramer MW, Hennenlotter J, Vetter G, Becker JU, Stenzl A, Merseburger AS, Schrader AJ, Kuczyk MA, Serth J. Fibronectin 1 mRNA expression correlates with advanced disease in renal cancer. *BMC Cancer* 2010; **10**: 503 [PMID: 20860816 DOI: 10.1186/1471-2407-10-503]
28 **Bittner M**, Meltzer P, Chen Y, Jiang Y, Seftor E, Hendrix M, Radmacher M, Simon R, Yakhini Z, Ben-Dor A, Sampas N, Dougherty E, Wang E, Marincola F, Gooden C, Lueders J, Glatfelter A, Pollock P, Carpten J, Gillanders E, Leja D, Dietrich K, Beaudry C, Berens M, Alberts D, Sondak V. Molecular classification of cutaneous malignant melanoma by gene expression profiling. *Nature* 2000; **406**: 536-540 [PMID: 10952317 DOI: 10.1038/35020115]
29 **Han S**, Ritzenthaler JD, Sitaraman SV, Roman J. Fibronectin increases matrix metalloproteinase 9 expression through activation of c-Fos *via* extracellular-regulated kinase and phosphatidylinositol 3-kinase pathways in human lung carcinoma cells. *J Biol Chem* 2006; **281**: 29614-29624 [PMID: 16882662 DOI: 10.1074/jbc.M604013200]
30 **Chung Y**, Law S, Kwong DL, Luk JM. Serum soluble E-cadherin is a potential prognostic marker in esophageal squamous cell carcinoma. *Dis Esophagus* 2011; **24**: 49-55 [PMID: 20807231 DOI: 10.1111/j.1442-2050.2010.01093.x]
31 **Bae KM**, Parker NN, Dai Y, Vieweg J, Siemann DW. E-cadherin plasticity in prostate cancer stem cell invasion. *Am J Cancer Res* 2011; **1**: 71-84 [PMID: 21968440]
32 **Kolle G**, Ho M, Zhou Q, Chy HS, Krishnan K, Cloonan N, Bertoncello I, Laslett AL, Grimmond SM. Identification of human embryonic stem cell surface markers by combined membrane-polysome translation state array analysis and immunotranscriptional profiling. *Stem Cells* 2009; **27**: 2446-2456 [PMID: 19650036 DOI: 10.1002/stem.182]
33 **Sethi S**, Macoska J, Chen W, Sarkar FH. Molecular signature of epithelial-mesenchymal transition (EMT) in human prostate cancer bone metastasis. *Am J Transl Res* 2010; **3**: 90-99 [PMID: 21139809]
34 **Zhang X**, Han B, Huang J, Zheng B, Geng Q, Aziz F, Dong Q. Prognostic significance of OCT4 expression in adenocarcinoma of the lung. *Jpn J Clin Oncol* 2010; **40**: 961-966 [PMID: 20462980 DOI: 10.1093/jjco/hyq066]
35 **Crnalic S**, Hörnberg E, Wikström P, Lerner UH, Tieva A, Svensson O, Widmark A, Bergh A. Nuclear androgen receptor staining in bone metastases is related to a poor outcome in prostate cancer patients. *Endocr Relat Cancer* 2010; **17**: 885-895 [PMID: 20688881 DOI: 10.1677/ERC-10-0059]
36 **Ni M**, Chen Y, Lim E, Wimberly H, Bailey ST, Imai Y, Rimm DL, Liu XS, Brown M. Targeting androgen receptor in estrogen receptor-negative breast cancer. *Cancer Cell* 2011; **20**: 119-131 [PMID: 21741601 DOI: 10.1016/j.ccr.2011.05.026]

P- Reviewer: Xu XC **S- Editor**: Wen LL
L- Editor: Kerr C **E- Editor**: Wu HL

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.1038

World J Gastroenterol 2014 January 28; 20(4): 1038-1047
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Fecal immunochemical test accuracy in average-risk colorectal cancer screening

Vicent Hernandez, Joaquin Cubiella, M Carmen Gonzalez-Mao, Felipe Iglesias, Concepción Rivera, M Begoña Iglesias, Lucía Cid, Ines Castro, Luisa de Castro, Pablo Vega, Jose Antonio Hermo, Ramiro Macenlle, Alfonso Martínez-Turnes, David Martínez-Ares, Pamela Estevez, Estela Cid, M Carmen Vidal, Angeles López-Martínez, Elisabeth Hijona, Marta Herreros-Villanueva, Luis Bujanda, Jose Ignacio Rodriguez-Prada; the COLONPREV study investigators

Vicent Hernandez, Felipe Iglesias, Lucía Cid, Luisa de Castro, Jose Antonio Hermo, Alfonso Martínez-Turnes, David Martínez-Ares, Pamela Estevez, M Carmen Vidal, Jose Ignacio Rodriguez-Prada; the COLONPREV study investigators, Department of Gastroenterology, Complexo Hospitalario Universitario de Vigo, 36200 Vigo, Spain

Joaquin Cubiella, Concepción Rivera, Ines Castro, Pablo Vega, Ramiro Macenlle, Estela Cid, Department of Gastroenterology, Complexo Hospitalario Universitario de Ourense, 32005 Ourense, Spain

M Carmen Gonzalez-Mao, Angeles López-Martínez, Department of Clinical Analysis, Complexo Hospitalario Universitario de Vigo, 36200 Vigo, Spain

M Begoña Iglesias, Department of Pathology, Complexo Hospitalario Universitario de Vigo, 36200 Vigo, Spain

Elisabeth Hijona, Marta Herreros-Villanueva, Luis Bujanda, Department of Gastroenterology, Donostia Hospital, Biodonostia Institute, University of the Basque Country UPV/EHU, CIBERehd, 20010 San Sebastian, Spain

Author contributions: Hernandez V is the guarantor of the article. Hernandez V, Cubiella J, Gonzalez-Mao MC and Iglesias F participated in the planning of the study; Hernandez V, Cubiella J, Gonzalez-Mao MC, Iglesias F, Rivera C, Iglesias MB, Cid L, Castro I, de Castro L, Vega P, Hermo JA, Macenlle R, Martínez-Turnes A, Martínez-Ares D, Estevez P, Cid E, Vidal MC, López-Martínez A, Hijona E, Herreros-Villanueva M, Bujanda L, Rodriguez-Prada JI took part in the execution of the study; Hernandez V, Cubiella J analyzed the study; Finally, all authors read the manuscript, made comments and approved the final version submitted.

Supported by Grants from the Conselleria de Sanidade of Xunta de Galicia, No. PS09/74; Asociación Española contra el Cáncer (Fundación Científica), Instituto de Salud Carlos III, No. PI08/90717; Obra Social de Kutxa, Diputación Foral de Gipuzkoa, No. DFG 07/5; Departamento de Sanidad del Gobierno Vasco, EITB-Maratoia, No. BIO 07/CA/19; Acción Transversal contra el Cáncer del CIBERehd (2008); CIBERehd funded by the Instituto de Salud Carlos III; and Dirección Xeral de Innovación e Xestión da Saúde Pública, Conselleria de Sanidade, Xunta de Galicia

Correspondence to: Joaquín Cubiella, MD, PhD, Department of Gastroenterology, Complexo Hospitalario Universitario de Ourense, C/ Ramón Puga 52-54, 32005 Ourense, Spain. joaquin.cubiella.fernandez@sergas.es
Telephone: +34-98-8385399 Fax: +34-98-8385399
Received: September 13, 2013 Revised: October 18, 2013
Accepted: November 12, 2013
Published online: January 28, 2014

Abstract

AIM: To assess the fecal immunochemical test (FIT) accuracy for colorectal cancer (CRC) and advanced neoplasia (AN) detection in CRC screening.

METHODS: We performed a multicentric, prospective, double blind study of diagnostic tests on asymptomatic average-risk individuals submitted to screening colonoscopy. Two stool samples were collected and the fecal hemoglobin concentration was determined in the first sample (FIT1) and the highest level of both samples (FITmax) using the OC-sensor™. Areas under the curve (AUC) for CRC and AN were calculated. The best FIT1 and FITmax cut-off values for CRC were determined. At this threshold, number needed to scope (NNS) to detect a CRC and an AN and the cost per lesion detected were calculated.

RESULTS: About 779 individuals were included. An AN was found in 97 (12.5%) individuals: a CRC in 5 (0.6%) and an advanced adenoma (≥ 10 mm, villous histology or high grade dysplasia) in 92 (11.9%) subjects. For CRC diagnosis, FIT1 AUC was 0.96 (95%CI: 0.95-0.98) and FITmax AUC was 0.95 (95%CI: 0.93-0.97). For AN, FIT1 and FITmax AUC were similar (0.72, 95%CI:

0.66-0.78 *vs* 0.73, 95%CI: 0.68-0.79, respectively, *P* = 0.34). Depending on the number of determinations and the positivity threshold cut-off used sensitivity for AN detection ranged between 28% and 42% and specificity between 91% and 97%. At the best cut-off point for CRC detection (115 ng/mL), the NNS to detect a CRC were 10.2 and 15.8; and the cost per CRC was 1814€ and 2985€ on FIT1 and FITmax strategies respectively. At this threshold the sensitivity, NNS and cost per AN detected were 30%, 1.76, and 306€, in FIT1 strategy, and 36%, 2.26€ and 426€, in FITmax strategy, respectively.

CONCLUSION: Performing two tests does not improve diagnostic accuracy, but increases cost and NNS to detect a lesion.

Key words: Colorectal neoplasms; Early detection of cancer; Sensitivity and specificity; Adenoma; Occult blood; Cost-benefit analysis

Core tip: Our study has determined fecal immunochemical test (FIT) diagnostic accuracy, number needed to scope and cost per lesion detected in colorectal cancer (CRC) screening programs. FIT is highly sensitive for CRC detection, allowing a drastic reduction in the cost per lesion detected when compared with direct screening colonoscopy. These data are relevant to design CRC screening programs in this setting.

Hernandez V, Cubiella J, Gonzalez-Mao MC, Iglesias F, Rivera C, Iglesias MB, Cid L, Castro I, de Castro L, Vega P, Hermo JA, Macenlle R, Martínez-Turnes A, Martínez-Ares D, Estevez P, Cid E, Vidal MC, López-Martínez A, Hijona E, Herreros-Villanueva M, Bujanda L, Rodriguez-Prada JI; the COLONPREV study investigators. Fecal immunochemical test accuracy in average-risk colorectal cancer screening. *World J Gastroenterol* 2014; 20(4): 1038-1047 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/1038.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.1038

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of cancer-related death[1]. Evidence of effectiveness of CRC screening in average-risk population is available from randomized controlled trials for guaiac fecal occult blood tests[2,3] and sigmoidoscopy[4,5], and it has been shown that it is cost-effective or even cost-saving[6].

Although guaiac fecal occult blood tests are effective in CRC screening, several drawbacks have been described: low sensitivity for advanced colorrectal neoplasia (AN) and need of diet and medication restriction[7]. In contrast, fecal immunochemical tests (FIT) are highly specific for detecting human blood of colonic origin[7], some use an automated analysis for reading test results[7], and they have shown a higher sensitivity and specificity for CRC and AN[8-14]. Despite the superiority of FIT over guaiac based methods, its accuracy in average-risk population screening and the optimal number of stool samples or cut-off level has not been properly assessed. To our knowledge, only four studies (performed on three different cohorts of patients) have assessed the FIT accuracy in average-risk patients who were screened with colonoscopy[13-16].

The COLONPREV study (ClinicalTrials.gov, NCT00906997), designed to compare the efficacy of one-time colonoscopy and biennial FIT for reducing CRC-related mortality at 10 years in asymptomatic, average-risk individuals, offered an ideal framework to develop diagnostic tests studies, as a group of individuals were randomly assigned to colonoscopy screening[17]. So, we performed a prospective, nested study on individuals invited to the COLONPREV study to assess the accuracy of FIT to detect CRC and AN, as well as to establish the optimal number of FIT, the best cut-off value for CRC detection, and the resource consumption per lesion detected.

MATERIALS AND METHODS

Study design

A multicentre, prospective, blinded, cohort study of diagnostic test was performed in three tertiary hospitals in Spain between 1st January 2010 and 30th June 2011, aiming to assess the accuracy of FIT for AN and CRC detection in average-risk population.

Study population

Asymptomatic men and women aged 50 to 69 years, included in the COLONPREV study in Galicia and Euskadi were invited to participate in this diagnostic test study if they were offered a colonoscopy during the inclusion period. Exclusion criteria have been described elsewhere[17] and included personal history of CRC, adenoma or inflammatory bowel disease, family history of hereditary or familial CRC (*i.e.*, > 2 first-degree relatives with CRC or one diagnosed before the age of 60 years), severe comorbidity, previous colectomy, FIT screening in the past 2 years, sigmoidoscopy or colonoscopy within the past 5 years, or symptoms requiring additional work-up. Individuals were also excluded if they did not accept the study or refused to undergo the colonoscopy.

Study interventions

All participants collected 2 stool samples from 2 consecutive days the week before the colonoscopy was scheduled. FIT was assessed using the automated OC-sensor™ (Eiken Chemical Co, Tokyo, Japan), without diet or medication restrictions. Samples were processed as previously described[18]. In each patient fecal hemoglobin (ng/mL of buffer), in the first sample (FIT1) and the highest level of

the two samples (FITmax) was determined. Laboratory staff were blinded for the colonoscopy result, and endoscopists performing the colonoscopy were blinded for the FIT result.

Bowel cleansing, sedation and colonoscopy procedure was performed according to the Spanish Guidelines on Quality of Colonoscopy in CRC Screening[19]. Polyps were categorized as non-neoplastic or neoplastic (adenomas). Adenomas of 10 mm or more in size, or with villous architecture (> 25%), or with high-grade dysplasia or intramucosal carcinoma were classified as advanced adenomas. Invasive cancer was considered when malignant cells were observed beyond the muscularis mucosa. Advanced colorectal neoplasia was defined as advanced adenoma or invasive cancer. Tumor staging was performed according to the AJCC classification[20]. Patients were classified according to the most advanced lesion.

Sample size calculation

Reported FIT sensitivity and specificity for AN in asymptomatic individuals was, respectively, 27.1% and 96.1%[21], while in patients undergoing a colonoscopy for any reason, these figures were 50.9%-67% and 88%-91.4%[22,23]. The prevalence of AN in average-risk, asymptomatic individuals ranges from 6.3% to 10.5%[24-27]. Taking these parameters into account, a sample size ranging from 724 to 1350 individuals would provide a 10% accuracy at a 5% bilateral significance level.

Analysis of resources and cost-benefit analysis

For each positivity threshold and strategy, the number of subjects needed to scope (NNS) to detect an AN or a CRC and the direct cost per lesion detected were determined. The analysis costs were calculated on the basis of the published colonoscopy costs in Spain (colonoscopy, 70€; colonoscopy with biopsy, 140€)[28] and FIT determination cost (3.2€).

Other aspects

The study was approved by the Galician Clinical Research Ethics Committee, under resolution dated 28th May 2009 (Code 2009/153). Patients' clinical histories were accessed for study purposes in accordance with the research protocols laid down by clinical documentation departments. All patients provided written informed consent.

Finally, to design the study and to write this original article the QADAS quality assessment tool for diagnostic tests, the STARD checklist and the STROBE checklist for cohort study were used[29-31].

Statistical analysis

The data were included in a specifically designed database (www.coloncruzer.es). Continuous variables were described using means and standard deviation, and categorical variables by the absolute number and percentage. Comparisons to identify differences in fecal hemoglobin concentrations between groups were performed using non-parametric tests (Mann-Whitney or Krukal-Wallis tests) in quantitative variables. To compare overall diagnostic accuracy for AN and CRC in both FIT1 and FITmax strategies the receiver operating characteristics (ROC) curve were drawn, and the χ^2 test for homogeneity of the corresponding area under the curve (AUC) was used. The best cut-off value of FIT1 and FITmax for CRC detection was determined with the Youden index. For each FIT testing strategy, sensitivity, specificity, positive and negative predictive value (PPV, NPV), as well as positive and negative likelihood ratio for the best cut-off and for prestablished positivity thresholds (50, 75, 100, 150 and 200 ng/mL) were calculated. Sensitivity and specificity for AN at the best CRC detection cut-off point was compared with the rest of thresholds using McNemar test[32].

The EPIDAT 3.1 software (Dirección Xeral de Innovación e Xestión da Saúde Pública, Santiago de Compostela, Spain) was used to perform sample size calculation, ROC curves drawings and comparisons. Statistical analyzes were performed using the SPSS statistical software, version 15.0 (SPSS Inc., Chicago, IL, United States). A P value < 0.05 was considered statistically significant.

RESULTS

Baseline characteristics

Overall, 851 subjects enrolled in the COLONPREV study were included in this FIT accuracy study. Fifty-four individuals did not complete the colonoscopy and 18 did not returned the FIT kit, so the evaluable population was 779 individuals: 386 male/393 female, mean age 57.55 ± 4.55 years. Hemoglobin concentration was 58.3 ± 278.4 ng/mL of buffer in the first determination and 57.3 ± 308.5 ng/mL in the second determination.

Invasive carcinoma was detected in 5 (0.6%) individuals (3 TNM Ⅰ; 1 TNM Ⅱ, 1 TNM Ⅲ), advanced adenomas in 92 (11.7%), and non advanced adenomas in 202 (25.9%). Therefore, AN was found in 97 (12.5%) patients. In 480 cases (61.6%) no neoplastic lesion was found; among them 124 had hiperplastic polyps, 6 had an inflammatory polyp, diverticula were found in 92 cases and an ulcerative colitis was detected in one patient.

Diagnostic accuracy of FIT

In patients with invasive CRC, FIT1 and FITmax (998 ± 1075.44, 1257.4 ± 1531.8) were significantly higher than in patients with advanced adenomas (233.14 ± 543.1 *vs* 325.3 ± 747.7, $P = 0.05$), non-advanced adenomas (42.4 ± 224.6 *vs* 76.7 ± 303.5, $P < 0.001$) or no neoplastic lesions (21.8 ± 150 *vs* 41.7 ± 216.5, $P < 0.001$). FIT1 and FITmax were similar in patients with non advanced adenomas and no neoplasms. Patients with CRC or AN had significantly higher FIT1 and FITmax than patients without these lesions (Figure 1).

Accuracy of FIT1 and FITmax was analyzed using ROC curves (Figure 2). For CRC diagnosis, the

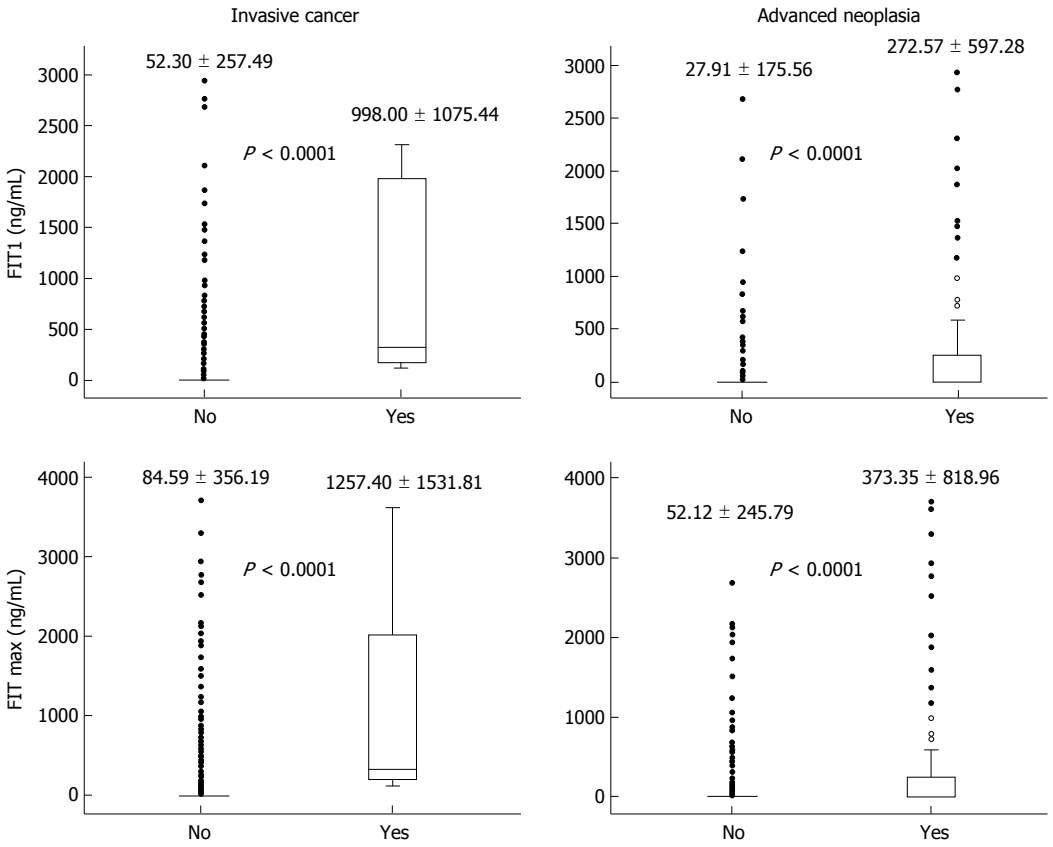


Figure 1 Fecal hemoglobin (ng/mL) according to the most advanced lesion. Values expressed as mean ± SD. Mann-Withney test. FIT: Fecal immunochemical test; FIT1: Fecal hemoglogin concentration in the first stool sample; FITmax: Highest fecal hemoglobin concentration of two stool samples.

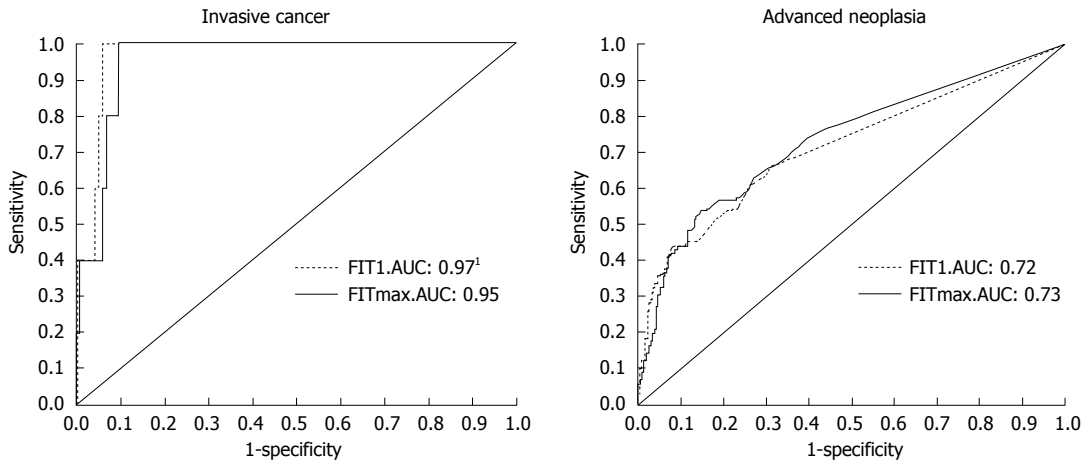


Figure 2 Receiver operating characteristics curves of fecal immunochemical test-1 and -max for advanced neoplasia and invasive cancer. FIT: Fecal immunochemical test; FIT1: Fecal hemoglogin concentration in the first stool sample; FITmax: Highest fecal hemoglobin concentration of two stool samples; AUC: Area under the curve. [1]*P* = 0.034 with respect to FITmax in the homogeneity area test.

AUC of FIT1 was 0.97 (95%CI: 0.94-0.99) and that of FITmax was 0.95 (95%CI: 0.92-0.99). The best cut-off value for CRC diagnosis was 115 ng/mL for both FIT1 and FITmax. For AN diagnosis the AUC of FIT1 was similar to that of FITmax (0.72, 95%CI: 0.66-0.77 *vs* 0.73, 95%CI: 0.67-0.79, respectively; homogeneity area test *P* = 0.27). The best cut-off value for AN diagnosis was 8 ng/mL in the FIT1 strategy and 20 ng/mL in the FITmax strategy.

Performance characteristics of FIT1 and FITmax

Table 1 describes sensitivity, specificity, PPV, NPV, LLR + and - to detect a CRC at different FIT1 and FITmax positivity thresholds. As shown, both strategies show a high sensitivity and specificity for CRC, although FITmax decreases specificity and PPV, without increasing

Table 1 Performance characteristics of fecal immunochemical test-1 and -max for colorectal cancer detection at different positive thresholds *n* (%)

Hemoglobin concentration (ng/mL)	FIT strategy	Individuals with a positive result[1]	Sensitivity[2]	Specificity[2]	Positive predictive value[2]	Negative predictive value[2]	Positive likelihood ratio[2]	Negative likelihood ratio[2]
50	FIT1[3]	67 (8.6)	100 (90-100)	92 (90-94)	7 (0-15)	100 (100-100)	12.48 (9.83-15.85)	-
	FITmax[4]	101 (13.0)	100 (90-100)	88.60 (85-90)	5 (0-10)	100 (100-100)	8.06 (6.69-9.72)	-
75	FIT1[3]	61 (7.8)	100 (90-100)	93 (91-95)	8 (0-16)	100 (100-100)	13.82 (10.74-17)	-
	FITmax[4]	89 (11.4)	100 (90-100)	89 (87-91)	6 (0-11)	100 (100-100)	9.21 (7.53-11.28)	-
100	FIT1[3]	55 (7.1)	100 (90-100)	94 (92-95)	9 (1-18)	100 (100-100)	15.48 (11.84-20.24)	-
	FITmax[4]	82 (10.5)	100 (90-100)	90 (88-92)	6 (0-12)	100 (100-100)	10.05 (8.13-12.43)	-
115	FIT1[3]	51 (6.5)	100 (90-100)	94 (92-96)	10 (1-19)	100 (100-100)	16.83 (12.71-22.7)	-
	FITmax[4]	79 (10.1)	100 (90-100)	90 (88-93)	6 (0-12)	100 (100-100)	10.46 (8.42-12.99)	-
150	FIT1[3]	48 (6.2)	80 (35-100)	94 (93-96)	8 (0-17)	100 (100-100)	14.07 (8.33-23.76)	0.21 (0.04-1.22)
	FITmax[4]	70 (9.0)	80 (35-100)	91 (89-94)	6 (0-12)	100 (100-100)	9.38 (5.72-15.40)	0.22 (0.04-1.26)
200	FIT1[3]	45 (5.8)	80 (35-100)	95 (93-96)	9 (0-18)	100 (100-100)	15.10 (8.89-25.66)	0.21 (0.04-1.22)
	FITmax[4]	59 (7.6)	80 (35-100)	93 (91-95)	7 (0-14)	100 (100-100)	11.26 (6.78-18.69)	0.22 (0.04-1.24)

[1]Values are expressed as absolute numbers and percentage; [2]Values are expressed as percentage and its 95%CI; [3]Fecal hemoglobin concentration in the first sample; [4]Higher fecal hemoglobin concentration of the two samples. FIT: Fecal immunochemical test.

Table 2 Performance characteristics of fecal immunochemical test-1 and -max for advanced neoplasia[1] detection at different thresholds

Hemoglobin concentration (ng/mL)	FIT strategy	Individuals with a positive result[2]	Sensitivity[3]	*P*[4]	Specificity[3]	*P*[5]	Positive predictive value[3]	Negative predictive value[3]	Positive likelihood ratio[3]	Negative likelihood ratio[3]
50	FIT1[6]	67 (8.6)	35 (25-45)	0.06	95 (93-97)	0.001	51 (38-63)	91 (89-93)	7.24 (4.72-11.13)	0.68 (0.59-0.79)
	FITmax[7]	101 (13.0)	42 (32-53)	0.03	91 (89-93)	< 0.001	41 (31-51)	92 (90-94)	4.98 (3.44-6.72)	0.63 (0.53-0.75)
75	FIT1[6]	61 (7.8)	33 (23-43)	0.2	96 (94-97)	0.01	52 (39-66)	91 (89-93)	7.76 (4.92-12.23)	0.70 (0.61-081)
	FITmax[7]	89 (11.4)	40 (30-51)	0.1	93 (91-95)	0.03	44 (33-55)	92 (89-94)	5.48 (3.82-7.87)	0.65 (0.55-0.76)
100	FIT1[6]	55 (7.1)	32 (22-42)	0.5	96 (95-98)	0.5	56 (42-70)	91 (89-93)	9.08 (5.57-14.80)	0.71 (0.61-0.81)
	FITmax[7]	82 (10.5)	37 (27-47)	1	93 (91-95)	0.5	44 (33-55)	91 (89-93)	5.50 (3.76-8.05)	0.67 (0.58-0.79)
115	FIT1[6]	51 (6.5)	30 (20-40)		97 (95-98)		57 (42-71)	91 (88-93)	9.27 (5.56-15.46)	0.72 (0.64-0.83)
	FITmax[7]	79 (10.1)	36 (26-46)		94 (92-95)		44 (33-56)	91 (89-93)	5.59 (3.79-8.26)	0.68 (0.59-0.79)
150	FIT1[6]	48 (6.2)	28 (18-37)	0.5	97 (96-98)	1	56 (41-71)	90 (88-93)	9.04 (5.33-15.34)	0.74 (0.66-0.84)
	FITmax[7]	70 (9.0)	32 (22-42)	0.1	94 (92-96)	0.06	44 (32-57)	91 (88-93)	5.59 (3.67-8.51)	0.72 (0.63-0.83)
200	FIT1[6]	45 (5.8)	28 (18-37)	0.5	97 (96-99)	0.1	60 (44-75)	90 (88-93)	10.55 (6.04-18.41)	0.74 (0.65-0.84)
	FITmax[7]	59 (7.6)	28 (18-37)	0.08	95 (94-97)	< 0.001	46 (32-59)	90 (88-93)	5.93 (3.72-9.45)	0.76 (0.67-0.86)

[1]Advanced neoplasia: advanced adenomas (adenoma > 1 cm in size, with high-grade dysplasia, or with villous component > 25) or colorectal cancer; [2]Values are expressed as absolute numbers and percentage; [3]Values are expressed as percentage and its 95%CI; [4]Significance of the sensitivity differences when compared with the optimal cut-off point in McNemar test. Differences with $P < 0.05$ are considered statistically significant; [5]Significance of the specificity differences when compared with the optimal cut-off point in McNemar test. Differences with $P < 0.05$ are considered statistically significant; [6]Fecal hemoglobin concentration in the first sample; [7]Higher fecal hemoglobin concentration of the two samples. FIT: Fecal immunochemical test.

the sensitivity or NPV. Table 2 describes sensitivity, specificity, PPV, NPV, LLR positive and negative to detect an AN at different FIT1 and FITmax cut-off points. Depending on the number of determinations and the positivity threshold cut-off used sensitivity for AN detection ranged between 28% and 42% and specificity between 91% and 97%. In only one determination strategy, statistically significant differences in specificity between 115 ng/mL, 50 ng/mL ($P = 0.001$) and 75 ng/mL ($P = 0.01$) were detected. In FITmax strategy, statistically significant differences in specificity were found between 115 ng/mL, 50 ng/mL ($P < 0.001$), 75 ng/mL ($P = 0.03$) and 200 ng/mL ($P < 0.001$). Finally, in FITmax strategy, we found statistically significant differences in sensitivity between 115 ng/mL, 50 ng/mL ($P = 0.03$) and 200 ng/mL ($P = 0.008$).

Cost-benefit analysis

When direct colonoscopy screening was analyzed, the NNS to detect a CRC and an AN were 155.8 and 8.2, respectively. The NNS to detect a CRC or an AN decreased from the lowest positivity threshold to the best cut-off value, and then rose again. At 115 ng/mL, in the FIT1 strategy, the NNS to detect a CRC and an AN were 10.2 and 1.76. At the same cut-off point, in FITmax strategy, the NNS to detect a CRC and an AN increased to 15.8 and 2.26 respectively, as shown in Table 3.

Cost-benefit analysis is displayed in Table 3. The cost per CRC and AN detected in the direct colonoscopy screening strategy was 16898€ and 889€. In contrast in the optimal cut-off point, cost per CRC detected was reduced between 89.2% and 82.3% and cost per AN detected was reduced between 65.6% and 52.1%, depend-

Table 3 Number of colonoscopies needed to detect one lesion and cost per lesion (€) according to positivity threshold and fecal immunochemical test testing strategy

Lesion	Positivity threshold (ng/mL)	No. need to scope		Cost per lesion detected (€)		Cost increment (€)
		FIT1	FITmax	FIT1	FITmax	
CRC	0	155.80		16898		
	50	13.40	20.20	2206	3489	58.16
	75	12.20	17.80	2038	3223	58.15
	100	11.00	16.40	1912	3055	59.78
	115	10.20	15.80	1814	2985	64.55
	150	12.00	17.50	2163	3451	59.55
	200	11.25	14.75	2075	3083	48.58
Advanced neoplasia[1]	0	8.20		889		
	50	1.97	2.46	317	425	34.07
	75	1.91	2.28	312	413	32.37
	100	1.77	2.28	302	424	40.40
	115	1.76	2.26	306	426	39.22
	150	1.78	2.26	312	445	42.63
	200	1.67	2.19	311	456	46.62

[1]Advanced neoplasia: advanced adenomas (adenoma > 1 cm in size with high-grade dysplasia or with villous component > 25) or colorectal cancer. FIT: Fecal immunochemical test; CRC: Colorectal cancer.

ing on the number of FIT determinations. Finally, using two FIT determinations increased cost per CRC detected between 48.58 and 64.55%, and cost per AN detected between 32.37% and 46.62% when compared with only one FIT determination.

DISCUSSION

In this diagnostic tests study we have assessed the accuracy of FIT to detect AN and CRC in an average-risk cohort, and have compared the performance characteristics, endoscopic resources needed and cost-benefit of two FIT testing strategies (one-day *vs* two-day sampling). FIT only detected 30%-36% of AN, although its accuracy to detect CRC was very high (100% sensitivity and 90%-94% specificity). Furthermore, two-day sampling strategy did not enhance FIT accuracy and increased resource consumption compared to one-day sampling.

Our study has several strengths. First, it was performed on average-risk individuals participating in a pragmatic, population-based CRC screening study[17], with all participants undergoing a colonoscopy. Second, it includes an estimation of direct costs, allowing us to perform a cost-benefit analysis.

Studies addressed to assess FIT accuracy by performing FIT and colonoscopy to all the participants are scant[13-16,21-23,33-37]; some were performed on patients scheduled for colonoscopy because of symptoms or increased risk of CRC[22,23,33,34], others were performed on asymptomatic patients but included subjects with family risk or younger than 50 years[21,35-37]. To our knowledge only four studies (performed on three different cohorts)[13-16] have assessed the accuracy of FIT in average-risk individuals who were offered colonoscopy as CRC screening strategy. A Korean study[13] and two German studies[14,15] were carried out in the setting of oportunistic screening. Recently, a Dutch study has assessed FIT accuracy in a cohort of individuals participating in a population-based screening study[16]. Our study was also carried out on asymptomatic average-risk individuals, participating in a population-based screening study[17], which would allow us to obtain relevant information for CRC screening programs. At the best cut-off value, with one-day FIT, we found that diagnostic accuracy for AN detection is similar to that found in the studies performed on average-risk individuals with a quantitative FIT[13-16]. With respect to CRC, we found a higher sensitivity when compared to previous studies[13,16], but this could be explained by the low number of CRC detected in our cohort.

The performance characteristics of FIT can be adapted to screening variables (prevalence of CRC, participation rates, endoscopic resources), by modifying positivity threshold or by analyzing several stool samples[13,15,33-37]. However, only three studies[13,15,16] have assessed the accuracy of FIT at several cut-off points in an average-risk screening study. Moreover, the study by Park *et al*[13] is the one that also analyzed more than one stool sample per patient. As previously reported, we found that sensitivity was higher at the lower positivity threshold and, conversely, specificity increased when increasing the positivity threshold. With respect to the number of FIT performed, we decided to analyze only two stool samples per patient, as the ongoing regional screening programs in our country test for one or two stool samples[38]. When comparing 1-d FIT with 2- or 3-d FIT, no clear benefit of several-day sampling has been described, except in a Japanese study in which a qualitative FIT was used[35]. Two studies conducted on referral cohorts (with symptomatic or high risk patients), in which FIT and colonoscopy was performed in all the participants, did not find superiority of 2- or 3-d sampling over single sampling[33,34]. Studies performed in screened average-risk population (that underwent colonoscopy only if FIT was positive), have shown that 2-d sampling could be superior to 1-sampling in different characteristics (depending on the criteria to consider a result as positive) at a particular cut-off level, but they also found that 1-d testing could perform as well as 2-d strategy by changing the threshold of positivity[39-43]. In the study by Park *et al*[13], AUC for CRC was better with three or two test than with only the first day FIT. In our cohort, however, the AUC for CRC was similar for FIT1 and FITmax, and this could be explained by the fact that all CRC in our cohort were detected with the FIT1 best cut-off.

Modifying the positivity threshold or the number of stool samples to be analyzed not only affect the FIT accuracy, but it also has a great impact on the colonoscopy workload and on the efficiency of the screening, as it influences the rate of patients with a positive test and the PPV. In our study we found that the positivity rate was higher (13%) with the FITmax strategy at the lowest positivity threshold. The same results are

found in studies that compared one-day FIT with 2 or 3-d FITmax strategy[33,34,39-41,43]. Finally, as the colonoscopy workload accounts for about 40% to 50% of total screening costs[44,45] modifying the positivity rate and the PPV has relevant consequences. Several studies have assessed this issue with controversial results. We decided to assess the cost benefit analysis by calculating the cost per detected lesion, and as in previous studies[37,46], intermediate thresholds were the most cost-effective for one-day and two-day sampling, being the most cost-effective strategy one-day sampling with a positivity threshold of 115 ng/mL. Our study does not pretend to compare cost-effectiveness among different screening strategies. In fact, we have only made a cost-benefit analysis inside a diagnostic test study. In fact, when cost-effectiveness is assessed by simulation models[47-49], in which screening and treatment costs are related to life-years gained, the most cost-effective strategies are those that allow to detect the greatest number of lesions (lowest positivity threshold and 2-d sampling), provided there is unlimited colonoscopy capacity[47].

As commented previously, FIT sensitivity for AN ranges between 28% and 42% according to the number of determinations and the cut-off point used. Although this is a limitation in the context of a diagnostic test we must be aware of two conditions that favours FIT as a screening test for CRC. First, this effect is diminished by the lower participation rate in the colonoscopy group than in the FIT group. Moreover, in a recently randomized controlled study, the first round of FIT screening detected about half the number of advanced adenomas that were detected by colonoscopy in the first round[17]. Besides, the recurrent nature of FIT screening may reduce the apparent advantage of colonoscopy. In a recently published studies after 4 rounds of CRC screening with FIT, the positive predictive value of the FIT for AN was 40% at the first round, and approximately 33% in the subsequent rounds[50].

Our study has several limitations. First, our sample size was near the lowest range to assess the true accuracy of FIT with a 10% accuracy. However, the prevalence of AN in our series was higher than previously reported, and with that prevalence, our sample size and the previously reported sensitivity and specificity[21], the accuracy achieved in this study was estimated in 8.82%. Second, the number of invasive cancer was low and all of them were detected by FIT1, and this could bias our results. Despite this, the accuracy for AN is similar to that reported previously in average-risk screening[13,15], which makes us consider our results reliable in this setting.

In conclusion, our study shows a low sensitivity of FIT to detect AN, but a high specificity. Its accuracy for CRC detection is high in the setting of average-risk CRC population. With respect to the number of samples, 2-d sampling does not improve the accuracy for CRC, but increases the sensitivity for AN detection, at the expense of increasing the direct costs per lesion detected.

ACKNOWLEDGMENTS

Clinical investigators: Galicia: Mª Belén Aguado, Ana Alonso, M Teresa Alves, Celia Cabalerio, Ana Belén Fernández, Javier Fernandez-Seara, Ana Cruz González, Mª Dolores González, Simoneta González, Pilar Iglesias, Ángeles López-Martinez, Carmen Méndez, Isabel Pérez, Carmen Portasany, Mar Rionda, Rosa Rodríguez, Manuel Rubio, Miriam Vázquez, José Ángel Vázquez, Pablo Vega, Mª Carmen Vidal. Basque Country: María E Alkiza, Jone Altzibar, Pilar Amiano, Juan Arenas, Edurne Artiñano, Ángel Cosme, Isabel Egitegi, Kepa Elorriaga, José L Elósegui, José M Enriquez-Navascués, Cristina Erce, Inés Gil, María A Gutiérrez-Stampa, Mariluz Jaúregui, Eva Laredo, Roberto Martínez, Maria J Mitxelena, Isabel Montalvo, Carlos Placer, Isabel Portillo, Cristina Sarasqueta. We acknowledge Dr. Enrique Quintero and Dr. Antoni Castells, national coordinators of the COLONPREV study, their support and valuable comments on the manuscript.

COMMENTS

Background

Colorectal cancer (CRC) is the third most common cancer worldwide and the second leading cause of cancer-related death. Evidence of effectiveness of CRC screening in average-risk population is available from randomized controlled trials for guaiac fecal occult blood tests and sigmoidoscopy, and it has been shown that it is cost-effective or even cost-saving.

Research frontiers

Despite the superiority of fecal immunochemical tests (FIT) over guaiac based methods, its accuracy in average-risk population screening and the optimal number of stool samples or cut-off level and the resource consumption per lesion detected has not been properly assessed.

Innovations and breakthroughs

In this diagnostic tests study authors have assessed the accuracy of FIT to detect advanced colorectal neoplasia and CRC in an average-risk cohort, and have compared the performance characteristics, endoscopic resources needed and cost-benefit of two FIT testing strategies (one-day *vs* two-day sampling). FIT only detected 30%-36% of advanced neoplasia (AN), although its accuracy to detect CRC was very high (100% sensitivity and 90%-94% specificity). Furthermore, two-day sampling strategy did not enhance FIT accuracy and increased resource consumption compared to one-day sampling.

Applications

Authors analyzed in this population different FIT strategies: 1 or 2 tests and different positive thresholds; not only to evaluate diagnostic accuracy but also endoscopic resources required and cost per lesion detected. Thus, analyzing two samples does not improve diagnostic accuracy and, instead, increases the costs by augmenting the number of colonoscopies needed to detect a CRC or an AN. Their cost-benefit analysis may allow health authorities to define the recommended strategy according to endoscopic resources.

Terminology

Average-risk population: Asymptomatic individuals aged 50-69 years with no familial history of CRC. Fecal immunochemical tests are based on the reaction of monoclonal or polyclonal antibodies specific for human hemoglobin, albumin or other fecal blood components.

Peer review

This is a multicentric study aimed at assessing accuracy of FIT in the detection of CRC and AN in patients undergoing CRC screening. The authors have compared specificity and sensitivity of two measures, one in the first sample and the other on the highest level of both samples. The authors showed a low sensitivity of FIT to detect AN, but a high specificity, which reach the highest level in the setting of average-risk CRC population. Two days sampling does

not improve the accuracy for CRC, but increases the sensitivity for AN detection even though is more expensive. The study is well designed and well written and the results are interesting.

REFERENCES

1 **Ferlay J**, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; **127**: 2893-2917 [PMID: 21351269 DOI: 10.1002/ijc.25516]

2 **Hewitson P**, Glasziou P, Watson E, Towler B, Irwig L. Cochrane systematic review of colorectal cancer screening using the fecal occult blood test (hemoccult): an update. *Am J Gastroenterol* 2008; **103**: 1541-1549 [PMID: 18479499 DOI: 10.1111/j.1572-0241.2008.01875.x]

3 **Shaukat A**, Mongin SJ, Geisser MS, Lederle FA, Bond JH, Mandel JS, Church TR. Long-term mortality after screening for colorectal cancer. *N Engl J Med* 2013; **369**: 1106-1114 [PMID: 24047060 DOI: 10.1056/NEJMoa1300720]

4 **Atkin WS**, Edwards R, Kralj-Hans I, Wooldrage K, Hart AR, Northover JM, Parkin DM, Wardle J, Duffy SW, Cuzick J; UK Flexible Sigmoidoscopy Trial Investigators. Once-only flexible sigmoidoscopy screening in prevention of colorectal cancer: a multicentre randomised controlled trial. *Lancet* 2010; **375**: 1624-1633 [PMID: 20430429 DOI: 10.1016/S0140-6736(10)60551-X]

5 **Schoen RE**, Pinsky PF, Weissfeld JL, Yokochi LA, Church T, Laiyemo AO, Bresalier R, Andriole GL, Buys SS, Crawford ED, Fouad MN, Isaacs C, Johnson CC, Reding DJ, O'Brien B, Carrick DM, Wright P, Riley TL, Purdue MP, Izmirlian G, Kramer BS, Miller AB, Gohagan JK, Prorok PC, Berg CD. Colorectal-cancer incidence and mortality with screening flexible sigmoidoscopy. *N Engl J Med* 2012; **366**: 2345-2357 [PMID: 22612596 DOI: 10.1056/NEJMoa1114635]

6 **Lansdorp-Vogelaar I**, Knudsen AB, Brenner H. Cost-effectiveness of colorectal cancer screening. *Epidemiol Rev* 2011; **33**: 88-100 [PMID: 21633092 DOI: 10.1093/epirev/mxr004]

7 **Quintero E**. Chemical or immunological tests for the detection of fecal occult blood in colorectal cancer screening? *Gastroenterol Hepatol* 2009; **32**: 565-576 [PMID: 19577340 DOI: 10.1016/j.gastrohep.2009.01.179]

8 **Smith A**, Young GP, Cole SR, Bampton P. Comparison of a brush-sampling fecal immunochemical test for hemoglobin with a sensitive guaiac-based fecal occult blood test in detection of colorectal neoplasia. *Cancer* 2006; **107**: 2152-2159 [PMID: 16998938 DOI: 10.1002/cncr.22230]

9 **Guittet L**, Bouvier V, Mariotte N, Vallee JP, Arsène D, Boutreux S, Tichet J, Launoy G. Comparison of a guaiac based and an immunochemical faecal occult blood test in screening for colorectal cancer in a general average risk population. *Gut* 2007; **56**: 210-214 [PMID: 16891354 DOI: 10.1136/gut.2006.101428]

10 **Allison JE**, Sakoda LC, Levin TR, Tucker JP, Tekawa IS, Cuff T, Pauly MP, Shlager L, Palitz AM, Zhao WK, Schwartz JS, Ransohoff DF, Selby JV. Screening for colorectal neoplasms with new fecal occult blood tests: update on performance characteristics. *J Natl Cancer Inst* 2007; **99**: 1462-1470 [PMID: 17895475 DOI: 10.1093/jnci/djm150]

11 **Rozen P**, Levi Z, Hazazi R, Waked A, Vilkin A, Maoz E, Birkenfeld S, Niv Y. Quantitative colonoscopic evaluation of relative efficiencies of an immunochemical faecal occult blood test and a sensitive guaiac test for detecting significant colorectal neoplasms. *Aliment Pharmacol Ther* 2009; **29**: 450-457 [PMID: 19035980 DOI: 10.1111/j.1365-2036.2008.03898.x]

12 **Parra-Blanco A**, Gimeno-García AZ, Quintero E, Nicolás D, Moreno SG, Jiménez A, Hernández-Guerra M, Carrillo-Palau M, Eishi Y, López-Bastida J. Diagnostic accuracy of immunochemical versus guaiac faecal occult blood tests for colorectal cancer screening. *J Gastroenterol* 2010; **45**: 703-712 [PMID: 20157748 DOI: 10.1007/s00535-010-0214-8]

13 **Park DI**, Ryu S, Kim YH, Lee SH, Lee CK, Eun CS, Han DS. Comparison of guaiac-based and quantitative immunochemical fecal occult blood testing in a population at average risk undergoing colorectal cancer screening. *Am J Gastroenterol* 2010; **105**: 2017-2025 [PMID: 20502450 DOI: 10.1038/ajg.2010.179]

14 **Hundt S**, Haug U, Brenner H. Comparative evaluation of immunochemical fecal occult blood tests for colorectal adenoma detection. *Ann Intern Med* 2009; **150**: 162-169 [PMID: 19189905 DOI: 10.7326/0003-4819-150-3-200902030-00005]

15 **Haug U**, Hundt S, Brenner H. Quantitative immunochemical fecal occult blood testing for colorectal adenoma detection: evaluation in the target population of screening and comparison with qualitative tests. *Am J Gastroenterol* 2010; **105**: 682-690 [PMID: 19953091 DOI: 10.1038/ajg.2009.668]

16 **de Wijkerslooth TR**, Stoop EM, Bossuyt PM, Meijer GA, van Ballegooijen M, van Roon AH, Stegeman I, Kraaijenhagen RA, Fockens P, van Leerdam ME, Dekker E, Kuipers EJ. Immunochemical fecal occult blood testing is equally sensitive for proximal and distal advanced neoplasia. *Am J Gastroenterol* 2012; **107**: 1570-1578 [PMID: 22850431 DOI: 10.1038/ajg.2012.249]

17 **Quintero E**, Castells A, Bujanda L, Cubiella J, Salas D, Lanas Á, Andreu M, Carballo F, Morillas JD, Hernández C, Jover R, Montalvo I, Arenas J, Laredo E, Hernández V, Iglesias F, Cid E, Zubizarreta R, Sala T, Ponce M, Andrés M, Teruel G, Peris A, Roncales MP, Polo-Tomás M, Bessa X, Ferrer-Armengou O, Grau J, Serradesanferm A, Ono A, Cruzado J, Pérez-Riquelme F, Alonso-Abreu I, de la Vega-Prieto M, Reyes-Melian JM, Cacho G, Díaz-Tasende J, Herreros-de-Tejada A, Poves C, Santander C, González-Navarro A. Colonoscopy versus fecal immunochemical testing in colorectal-cancer screening. *N Engl J Med* 2012; **366**: 697-706 [PMID: 22356323 DOI: 10.1056/NEJMoa1108895]

18 **Vilkin A**, Rozen P, Levi Z, Waked A, Maoz E, Birkenfeld S, Niv Y. Performance characteristics and evaluation of an automated-developed and quantitative, immunochemical, fecal occult blood screening test. *Am J Gastroenterol* 2005; **100**: 2519-2525 [PMID: 16279909 DOI: 10.1111/j.1572-0241.2005.00231.x]

19 **Jover R**, Herráiz M, Alarcón O, Brullet E, Bujanda L, Bustamante M, Campo R, Carreño R, Castells A, Cubiella J, García-Iglesias P, Hervás AJ, Menchén P, Ono A, Panadés A, Parra-Blanco A, Pellisé M, Ponce M, Quintero E, Reñé JM, Sánchez del Río A, Seoane A, Serradesanferm A, Soriano Izquierdo A, Vázquez Sequeiros E. Clinical practice guidelines: quality of colonoscopy in colorectal cancer screening. *Endoscopy* 2012; **44**: 444-451 [PMID: 22438159 DOI: 10.1055/s-0032-1306690]

20 **O'Connell JB**, Maggard MA, Ko CY. Colon cancer survival rates with the new American Joint Committee on Cancer sixth edition staging. *J Natl Cancer Inst* 2004; **96**: 1420-1425 [PMID: 15467030 DOI: 10.1093/jnci/djh275]

21 **Morikawa T**, Kato J, Yamaji Y, Wada R, Mitsushima T, Shiratori Y. A comparison of the immunochemical fecal occult blood test and total colonoscopy in the asymptomatic population. *Gastroenterology* 2005; **129**: 422-428 [PMID: 16083699 DOI: 10.1053/j.gastro.2005.05.056]

22 **Levi Z**, Rozen P, Hazazi R, Vilkin A, Waked A, Maoz E, Birkenfeld S, Leshno M, Niv Y. A quantitative immunochemical fecal occult blood test for colorectal neoplasia. *Ann Intern Med* 2007; **146**: 244-255 [PMID: 17310048 DOI: 10.7326/0003-4819-146-4-200702200-00003]

23 **Greenberg PD**, Bertario L, Gnauck R, Kronborg O, Hardcastle JD, Epstein MS, Sadowski D, Sudduth R, Zuckerman GR, Rockey DC. A prospective multicenter evaluation of new fecal occult blood tests in patients undergoing colonoscopy.

Am J Gastroenterol 2000; **95**: 1331-1338 [PMID: 10811348 DOI: 10.1111/j.1572-0241.2000.02032.x]

24 **Strul H**, Kariv R, Leshno M, Halak A, Jakubowicz M, Santo M, Umansky M, Shirin H, Degani Y, Revivo M, Halpern Z, Arber N. The prevalence rate and anatomic location of colorectal adenoma and cancer detected by colonoscopy in average-risk individuals aged 40-80 years. *Am J Gastroenterol* 2006; **101**: 255-262 [PMID: 16454827 DOI: 10.1111/j.1572-0241.2006.00430.x]

25 **Terhaar Sive Droste JS**, Craanen ME, van der Hulst RW, Bartelsman JF, Bezemer DP, Cappendijk KR, Meijer GA, Morsink LM, Snel P, Tuynman HA, van Wanrooy RL, Wesdorp EI, Mulder CJ. Colonoscopic yield of colorectal neoplasia in daily clinical practice. *World J Gastroenterol* 2009; **15**: 1085-1092 [PMID: 19266601 DOI: 10.3748/wjg.15.1085]

26 **Betés Ibáñez M**, Muñoz-Navas MA, Duque JM, Angós R, Macías E, Súbtil JC, Herraiz M, de la Riva S, Delgado-Rodríguez M, Martínez-Gonzélez MA. Diagnostic value of distal colonic polyps for prediction of advanced proximal neoplasia in an average-risk population undergoing screening colonoscopy. *Gastrointest Endosc* 2004; **59**: 634-641 [PMID: 15114305 DOI: 10.1016/S0016-5107(04)00155-5]

27 **Lieberman DA**, Weiss DG, Bond JH, Ahnen DJ, Garewal H, Chejfec G. Use of colonoscopy to screen asymptomatic adults for colorectal cancer. Veterans Affairs Cooperative Study Group 380. *N Engl J Med* 2000; **343**: 162-168 [PMID: 10900274 DOI: 10.1056/NEJM200007203430301]

28 **Ladabaum U**, Ferrandez A, Lanas A. Cost-effectiveness of colorectal cancer screening in high-risk Spanish patients: use of a validated model to inform public policy. *Cancer Epidemiol Biomarkers Prev* 2010; **19**: 2765-2776 [PMID: 20810603 DOI: 10.1158/1055-9965.EPI-10-0530]

29 **Whiting P**, Rutjes AW, Dinnes J, Reitsma J, Bossuyt PM, Kleijnen J. Development and validation of methods for assessing the quality of diagnostic accuracy studies. *Health Technol Assess* 2004; **8**: iii, 1-234 [PMID: 15193208 DOI: 10.3310/hta8250]

30 **Bossuyt PM**, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, Lijmer JG, Moher D, Rennie D, de Vet HC. Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. *BMJ* 2003; **326**: 41-44 [PMID: 12511463 DOI: 10.1136/bmj.326.7379.41]

31 **von Elm E**, Altman DG, Egger M, Pocock SJ, Gøtzsche PC, Vandenbroucke JP; STROBE Initiative. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. *J Clin Epidemiol* 2008; **61**: 344-349 [PMID: 18313558 DOI: 10.1016/j.jclinepi.2007.11.008]

32 **Trajman A**, Luiz RR. McNemar chi2 test revisited: comparing sensitivity and specificity of diagnostic examinations. *Scand J Clin Lab Invest* 2008; **68**: 77-80 [PMID: 18224558 DOI: 10.1080/00365510701666031]

33 **Rozen P**, Comaneshter D, Levi Z, Hazazi R, Vilkin A, Maoz E, Birkenfeld S, Niv Y. Cumulative evaluation of a quantitative immunochemical fecal occult blood test to determine its optimal clinical use. *Cancer* 2010; **116**: 2115-2125 [PMID: 20186820 DOI: 10.1002/cncr.25012]

34 **Oort FA**, van Turenhout ST, Coupé VM, van der Hulst RW, Wesdorp EI, Terhaar sive Droste JS, Larbi IB, Kanis SL, van Hengel E, Bouman AA, Meijer GA, Mulder CJ. Double sampling of a faecal immunochemical test is not superior to single sampling for detection of colorectal neoplasia: a colonoscopy controlled prospective cohort study. *BMC Cancer* 2011; **11**: 434 [PMID: 21985604 DOI: 10.1186/1471-2407-11-434]

35 **Nakama H**, Yamamoto M, Kamijo N, Li T, Wei N, Fattah AS, Zhang B. Colonoscopic evaluation of immunochemical fecal occult blood test for detection of colorectal neoplasia. *Hepatogastroenterology* 1995; **46**: 228-231 [PMID: 10228797]

36 **Nakama H**, Zhang B, Fattah AS. A cost-effective analysis of the optimum number of stool specimens collected for immunochemical occult blood screening for colorectal cancer. *Eur J Cancer* 2000; **36**: 647-650 [PMID: 10738130 DOI: 0959-8049/00/S]

37 **Nakama H**, Zhang B, Zhang X. Evaluation of the optimum cut-off point in immunochemical occult blood testing in screening for colorectal cancer. *Eur J Cancer* 2001; **37**: 398-401 [PMID: 11239763 DOI: 0959-8049/01/S]

38 **Salas D**. Situacion de los programas de cribado de cáncer colorrectal en España - 2012. Available from: URL: http://www.programascancerdemama.org/index.php/situacion-de-los-programas-de-cribado-de-cancer-colorrectal-en-espana-ano-2012. Accessed March 25, 2013

39 **Guittet L**, Bouvier V, Mariotte N, Vallee JP, Levillain R, Tichet J, Launoy G. Performance of immunochemical faecal occult blood test in colorectal cancer screening in average-risk population according to positivity threshold and number of samples. *Int J Cancer* 2009; **125**: 1127-1133 [PMID: 19431212 DOI: 10.1002/ijc.24407]

40 **Grazzini G**, Visioli CB, Zorzi M, Ciatto S, Banovich F, Bonanomi AG, Bortoli A, Castiglione G, Cazzola L, Confortini M, Mantellini P, Rubeca T, Zappa M. Immunochemical faecal occult blood test: number of samples and positivity cutoff. What is the best strategy for colorectal cancer screening? *Br J Cancer* 2009; **100**: 259-265 [PMID: 19142185 DOI: 10.1038/sj.bjc.6604864]

41 **van Roon AH**, Wilschut JA, Hol L, van Ballegooijen M, Reijerink JC, 't Mannetje H, Kranenburg LJ, Biermann K, van Vuuren AJ, Francke J, van der Togt AC, Habbema DJ, van Leerdam ME, Kuipers EJ. Diagnostic yield improves with collection of 2 samples in fecal immunochemical test screening without affecting attendance. *Clin Gastroenterol Hepatol* 2011; **9**: 333-339 [PMID: 21185397 DOI: 10.1016/j.cgh.2010.12.012]

42 **Guittet L**, Bouvier V, Guillaume E, Levillain R, Ruiz A, Lantieri O, Launoy G. Colorectal cancer screening: why immunochemical faecal occult blood test performs as well with either one or two samples. *Dig Liver Dis* 2012; **44**: 694-699 [PMID: 22525156 DOI: 10.1016/j.dld.2012.03.005]

43 **Faivre J**, Dancourt V, Manfredi S, Denis B, Durand G, Gendre I, Bidan JM, Jard C, Levillain R, Jung S, Viguier J, Dorval E. Positivity rates and performances of immunochemical faecal occult blood tests at different cut-off levels within a colorectal cancer screening programme. *Dig Liver Dis* 2012; **44**: 700-704 [PMID: 22542582 DOI: 10.1016/j.dld.2012.03.015]

44 **Castiglione G**, Zappa M, Grazzini G, Sani C, Mazzotta A, Mantellini P, Ciatto S. Cost analysis in a population based screening programme for colorectal cancer: comparison of immunochemical and guaiac faecal occult blood testing. *J Med Screen* 1997; **4**: 142-146 [PMID: 9368871 DOI: 10.1177/096914139700400306]

45 **Grazzini G**, Ciatto S, Cislaghi C, Castiglione G, Falcone M, Mantellini P, Zappa M. Cost evaluation in a colorectal cancer screening programme by faecal occult blood test in the District of Florence. *J Med Screen* 2008; **15**: 175-181 [PMID: 19106257 DOI: 10.1258/jms.2008.008032]

46 **Berchi C**, Guittet L, Bouvier V, Launoy G. Cost-effectiveness analysis of the optimal threshold of an automated immunochemical test for colorectal cancer screening: performances of immunochemical colorectal cancer screening. *Int J Technol Assess Health Care* 2010; **26**: 48-53 [PMID: 20059780 DOI: 10.1017/S0266462309990808]

47 **Wilschut JA**, Habbema JD, van Leerdam ME, Hol L, Lansdorp-Vogelaar I, Kuipers EJ, van Ballegooijen M. Fecal occult blood testing when colonoscopy capacity is limited. *J Natl Cancer Inst* 2011; **103**: 1741-1751 [PMID: 22076285 DOI: 10.1093/jnci/djr385]

48 **Wilschut JA**, Hol L, Dekker E, Jansen JB, Van Leerdam ME, Lansdorp-Vogelaar I, Kuipers EJ, Habbema JD, Van Ballegooijen M. Cost-effectiveness analysis of a quantitative

immunochemical test for colorectal cancer screening. *Gastroenterology* 2011; **141**: 1648-1655.e1 [PMID: 21784045 DOI: 10.1053/j.gastro.2011.07.020]

49 **Goede SL**, van Roon AH, Reijerink JC, van Vuuren AJ, Lansdorp-Vogelaar I, Habbema JD, Kuipers EJ, van Leerdam ME, van Ballegooijen M. Cost-effectiveness of one versus two sample faecal immunochemical testing for colorectal cancer screening. *Gut* 2013; **62**: 727-734 [PMID: 22490518 DOI: 10.1136/gutjnl-2011-301917]

50 **Crotta S**, Segnan N, Paganin S, Dagnes B, Rosset R, Senore C. High rate of advanced adenoma detection in 4 rounds of colorectal cancer screening with the fecal immunochemical test. *Clin Gastroenterol Hepatol* 2012; **10**: 633-638 [PMID: 22426085 DOI: 10.1016/j.cgh.2012.02.030]

P- Reviewers: Franceschi F, Hsiao KCW, Hardt PD, Kita H, Maurel J, Triantafyllou K
S- Editor: Gou SX **L- Editor**: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.1048

World J Gastroenterol 2014 January 28; 20(4): 1048-1053
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Biopsy-driven diagnosis in infants with cholestatic jaundice in Iran

Elham Talachian, Ali Bidari, Mitra Mehrazma, Nahid Nick-khah

Elham Talachian, Nahid Nick-khah, Department of Pediatric Gastroenterology, Ali Asghar Children's Hospital, Iran University of Medical Sciences, Tehran 1919816766, Iran
Ali Bidari, Rasoul Akram General Hospital, Iran University of Medical Sciences, Tehran 1445613131, Iran
Mitra Mehrazma, Oncopathology Research Center, Iran University of Medical Sciences, Tehran 1449614525, Iran
Author contributions: Talachian E performed the majority of the experiments, data analysis and interpretation; Bidari A contributed to performing the experiments, data analysis and writing and editing of the manuscript; Mehrazama M was involved in designing the proposal as well as reporting results of biopsy specimens and editing the manuscript; Nick-khah N was responsible for chart reviews, extracting and analyzing the data, and was involved in providing the primary draft of the manuscript.
Correspondence to: Ali Bidari, MD, Rasoul Akram General Hospital, Iran University of Medical Sciences, Niyayesh St., Sattarkhan Ave., Tehran 1445613131, Iran. bidari.a@iums.ac.ir
Telephone: +98-912-3277847 Fax: +98-21-66525327
Received: August 13, 2013 Revised: November 7, 2013
Accepted: November 18, 2013
Published online: January 28, 2014

Abstract

AIM: To determine the frequencies of diagnoses confirmed by liver biopsy in infants with cholestasis in an Iranian pediatric hospital.

METHODS: This was a retrospective study conducted in a tertiary referral children's hospital in Iran. We retrieved all pathology reports of liver biopsies from children less than two years of age who had presented for evaluation of cholestatic jaundice from March 2001 to March 2011. Additional specimen samples obtained from archived pathology blocks were reviewed by a pathologist blinded to the final diagnosis. These results were compared with the pathology reports from chart records to ensure consensus and eliminate any inconsistencies in final diagnoses. A structured checklist was used to gather information on multiple variables including age, sex, gestational age at birth, birth weight, age at which hyperbilirubinemia manifested, presence and identification of associated anomalies, clinical manifestations, and histological findings from liver biopsies. The baseline data are reported using descriptive statistics, and differences between groups were assessed by Fisher's exact test and Student's *t* test when indicated.

RESULTS: Fifty-five cases (28 females; 27 males) of infantile cholestasis (IC) were included in this study. The mean serum total bilirubin and direct bilirubin at presentation were 13.6 ± 5.9 and 7.3 ± 3.4, respectively. Forty cases (72.7%) were the product of term pregnancies. Common associated clinical findings were acholic stool in 33 cases (60.0%), hepatomegaly in 30 cases (54.5%), and dark-colored urine in 21 cases (38.2%). Biliary atresia (BA) was the most frequent diagnosis, found in 32 cases (58.2%), followed by intrahepatic bile duct paucity found in 6 cases (10.9%), metabolic disease in 6 cases (10.9%), idiopathic neonatal hepatitis in 5 cases (9.1%), choledochal cyst in 2 cases (3.6%), liver cirrhosis in 2 cases (3.6%), and progressive familial intrahepatic cholestasis and portal fibrosis each in 1 case (1.8%). The mean times for jaundice onset and liver biopsy were 43.8 and 102.0 d, respectively. In BA, the mean age at jaundice presentation was 21 d and for liver biopsy was 87.5 d, representing a mean delay of 66.5 d.

CONCLUSION: A significant delay was found between IC presentation and liver biopsy, which is detrimental in conditions that can cause irreversible liver damage, such as BA.

Key words: Cholestasis; Neonate; Hepatitis; Biliary atresia; Neonatal hepatitis; Infant; Conjugated hyperbilirubinemia; Liver biopsy

Core tip: Infantile cholestasis is a heterogeneous disor-

der characterized by abnormal direct hyperbilirubinemia after the second week of life. While biliary atresia (BA), progressive familial intrahepatic cholestasis, and idiopathic neonatal hepatitis are among the most prevalent causes, BA specifically needs early surgical intervention to avoid cirrhosis. This makes liver biopsy a crucial procedure for timely surgical consideration. We found that there was a significant delay from the time that jaundice was noted to the time of liver biopsy in those eventually diagnosed with BA. These results demonstrate that an early diagnostic approach is prudent to avoid irreversible hepatic complications.

Talachian E, Bidari A, Mehrazma M, Nick-khah N. Biopsy-driven diagnosis in infants with cholestatic jaundice in Iran. *World J Gastroenterol* 2014; 20(4): 1048-1053 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/1048.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.1048

INTRODUCTION

Jaundice, also known as icterus, is a common heterogeneous condition in neonates that usually resolves by the end of the second week after birth[1]. Icterus lasting beyond 2 wk of life, and especially if it is of the conjugated type, is perceived as clinically significant[2,3]. Conjugated serum bilirubin is considered abnormal if measured more than 1 mg/dL when total bilirubin is less than 5 mg/dL, or more than 20% of the total bilirubin in cases of more severe hyperbilirubinemia[3-5].

Cholestasis caused by diminished canalicular bile flow is clinically characterized by persistent conjugated hyperbilirubinemia[5]. Anatomical approaches to diagnosing cholestatic jaundice, which categorize the causes as either intrahepatic or extrahepatic, can be useful in the clinical setting, however not all conditions, including biliary atresia (BA), fit into a single category. Therefore, a more plausible classification separates the causes into functional and structural types. Using this strategy, functional derangements include metabolic, infectious, toxic, hemodynamic and idiopathic insults and structural abnormalities encompass biliary atresia, choledochal cysts and bile duct strictures[6].

BA usually presents in the first few weeks of life[7]. Progressive familial intrahepatic cholestasis (PFIC) begins in infancy with a mean age for jaundice onset of about 3 mo, although some patients do not develop jaundice until much later, even into adolescence[6]. The common causes of infantile cholestasis vary with age of onset, with the relative frequency of any individual diagnosis shifting when moving from a neonatal to a late infancy period[5,6].

When evaluating infants with conjugated hyperbilirubinemia, liver biopsy is the most reliable and definitive procedure[4,5]. Liver histopathology provides important clues to the correct diagnosis with a diagnostic yield as high as 95%[5,6,8]. Specimens displaying a proliferation of bile ducts, biliary plugs, portal tract edema and fibrosis suggest BA, while derangement in lobular architecture and ballooning of hepatocytes in association with focal hepatic necrosis along with the presence of multinucleated giant cells is highly indicative of neonatal hepatitis[6,9,10]. In practice, the main purpose for liver biopsies in infants with cholestasis is to define whether biliary obstruction is present or not. Furthermore, a liver biopsy can help with the determination of the severity of hepatocellular injury and assessment of the prognosis[6,8].

The literature suggests that since 1970, there has been a shift from identifying idiopathic neonate hepatitis (INH) as the most common cause of cholestasis to more clearly defined disorders such as PFIC and bile acid synthetic defects[6]. Unfortunately, there is a paucity of data on the prevalence of diverse etiologies of cholestasis in non-western countries, especially in the middle east area[11-13]. This study was conducted to determine the frequency of different diagnoses confirmed by liver biopsy in infants with cholestasis admitted to a pediatric hospital in Iran.

MATERIALS AND METHODS

In this retrospective study, we retrieved all pathology reports on liver specimens from children of less than two years age who were admitted to the Ali-Asghar Children's Hospital between March 2001 and March 2011. The corresponding hospital files were reviewed and those with cholestatic jaundice identified. A structured checklist was used to gather information on: age, sex, gestational age at birth, birth weight, age at which hyperbilirubinemia manifested, presence of associated anomalies, clinical manifestations, and histological findings from liver biopsies. The final diagnoses were obtained from hospital files. Original pathology reports were compared with the study pathologist's interpretation and both were correlated with a final diagnosis to avoid inconsistencies. SPSS version 18 statistical software was used to analyze the data. Descriptive statistics were employed to report frequencies and means ± SD. To show differences between groups we used a Fisher's exact test and Student's *t* test as indicated; $P < 0.05$ was considered significant.

RESULTS

In total, 55 infants with cholestatic jaundice and available biopsy reports were entered into the study. Twenty-eight (51%) were female and 27 (49%) were male. Although the mean time for the onset of jaundice was 43.8 d, the mean time for taking liver biopsy was 102 d after birth, representing a notable delay in attempting to perform diagnostic liver biopsy. The baseline clinical and laboratory characteristics can be found in Table 1.

Liver biopsies from 32 infants had firm histopathological evidence of BA, making it the most common cause of cholestasis, with no significant sex difference ($P = 0.45$). Twenty-four (75%) of these BA cases were in infants from term pregnancies with normal birth weights. The next most frequent diagnoses were paucity of bile ducts, metabolic disorders, and INH (Table 2). The pau-

Table 1 Clinical and laboratory characteristics of the 55 infants with cholestasis included for analysis of biopsy diagnosis

Variable	Mean ± SD	Range
Gestational age (wk)	38.4 ± 2.7	31-41
Birth weight (g)	2785 ± 658	1300-3980
Age when jaundice came to clinical attention (d)	43.8 ± 93	1-630
Age at liver biopsy (d)	102 ± 110	8-690
Total bilirubin (mg/dL)	13.6 ± 5.9	3.9-36
Direct bilirubin (mg/dL)	7.3 ± 3.4	2.4-19
Alkaline phosphatase (IU/L)	1244 ± 800	9.5-3379
AST (IU/L)	280 ± 223	36-1009
ALT (IU/L)	238 ± 486	16-3510
GGT (IU/L)	412 ± 508	35-1981
PT (s)	14.6 ± 4.5	11-38
PTT (s)	39.2 ± 10	26-82
Albumin (g/dL)	3.5 ± 0.9	1.3-5.4
Hgb (g/dL)	10 ± 1.9	6.9-16.4
Platelet × 10^3/μL	381 ± 164	76-700

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transpeptidase; Hgb: Hemoglobin; PT: Prothrombin time; PTT: Partial thromboplastin time.

city of bile ducts was an isolated finding in five of the six observed cases, however one male had Alagille's syndrome, characterized by a ventricular septal defect, hypertelorism, a prominent forehead and cholestatic jaundice. Six cases were affected by metabolic disease, comprised of five cases with glycogen storage disease and one case with galactosemia. Another frequent cause of conjugated hyperbilirubinemia in our series was INH, affecting 5 infants (9%). Three of these were premature with a birth weight of less than 2500 g.

Frequencies for a set of selected variables for individual diagnoses (including sex, birth weight, perinatal findings, clinical findings and jaundice course) are shown in Table 3. Jaundice was invariably present and acholic stool, hepatomegaly and dark-colored urine were all common. Reported in Table 4 are the frequencies of some laboratory findings including hemoglobin, platelet and liver function tests for different disease entities. The highest levels of hyperbilirubinemia were detected in PFIC with a mean value of 28 mg/dL followed by BA with a mean of 14.4. Alkaline phosphatase levels were elevated in all diagnostic categories without statistically significant differences between the subgroups. Levels of gamma-glutamyl transpeptidase, however, were significantly higher in cases with BA (Fisher's exact test, $P < 0.05$). Coagulopathy was seen in several subgroups, but the lone abnormal coagulation test with a significant prediction for a specific group was the partial thromboplastin time (PTT), which was abnormal in those proved to have cirrhosis by liver biopsy (Fisher's exact test, $P < 0.05$).

DISCUSSION

The results of this study show that BA was the most common cause of cholestasis, identified in 58% of the liver biopsies examined. BA is the most common cause of prolonged cholestatic jaundice in neonates and accounts for 40%-50% of all pediatric liver transplants[4,6,7]. The majority of infants diagnosed with BA in our study had normal birth weights from term pregnancies, in agreement with results from Mowat *et al*[14], showing that only one infant out of 32 had been born prematurely. A tendency for female preponderance was noted a tendency for female preponderance was noted (Table 3). All cases displayed jaundice, with common occurrences of acholic stool, hepatomegaly and dark colored urine, consistent with previous reports in the literature[4,13-15]. While congenital malformations have been reported in one-third of BA cases[16,17], only 13% of the infants included in this study had congenital anomalies.

INH is a differential diagnosis for BA, as both conditions are characterized by conjugated hyperbilirubinemia presented early after birth and are associated with hepatomegaly. In this study, five cases of INH were identified, all of which displayed hepatomegaly, which is often a presenting feature of liver disease. Three of the infants diagnosed with INH were premature and of low birth weight, two features that are frequently reported in INH[4,14]. Interestingly, two infants had acholic stools. Although acholic stool is a cardinal feature of biliary obstruction, it may also occur as a result of severe bile secretory failure at the level of the hepatocyte[14]. Thus, liver biopsy is required for accurate diagnosis in most cases[4,7,8,18-20].

While BA and INH cannot be distinguished based on clinical features, such as hepatomegaly, splenomegaly, coagulopathy or acholic stool[7,14,19], they can differ with regard to their course, prognosis and management[4,18]. While INH has a variable and sometimes a self-limiting course[17,18], BA is a relentlessly progressive disease requiring surgical intervention and/or liver transplantation[4,16,21]. In our case series, all of the five INH cases had progressive jaundice before liver biopsy. However, our selected population was confined to those who underwent liver biopsy, therefore we assume a selection bias which could have caused overrepresentation of a subgroup of INH cases whose cholestasis progressed such that a biopsy procedure was attempted. Noteworthy, there was a mean time lapse of 66.5 d in BA and 55 d in INH from the time jaundice first came to clinical attention to the time that diagnostic liver biopsy was performed, in comparison to a previous study reporting a mean delay of 120.8 d in BA and 65.9 d in INH[22].

Since unmanaged biliary atresia may result in cirrhosis within a few weeks, a prompt and accurate diagnosis is of outmost importance[23]. Therefore, neonatal cholestasis beyond the second week of life should be considered as a serious condition that needs urgent investigation and possible liver biopsy[5,24,25]. Causes for delayed intervention, as identified in a study by Mieli-Vergani *et al*[26], include a lack for follow-up of neonatal jaundice, inadequate investigation of hemorrhagic disease, misdiagnosis of breast milk jaundice, pigmented stools and decreased serum bilirubin. For many cases, delayed recognition and referral for specialty care remain major barriers to timely

Table 2 Diagnoses from liver biopsies and their frequencies in cases of infantile cholestasis *n* (%)

Biopsy findings	Frequency	Mean gestational age in weeks	Mean birth weight in grams	Mean age when jaundice came to clinical attention (range)	Mean age at liver biopsy in days
BA	32 (58)	38.4	2850	21 (1-120)	87.5
Paucity of bile ducts	6 (11)	39.0	2600	20.5 (1-60)	59
Metabolic disease	6 (11)	39.4	3380	221 (44-630)	266
INH	5 (9)	36.4	2230	38 (3-90)	93
Choledochal cyst	2 (3.6)	40.0	2750	20 (15-26)	82
Liver cirrhosis	2 (3.6)	38.5	1950	10 (9-13)	34
PFIC	1 (1.8)	40.0	3300	135	165
Portal fibrosis	1 (1.8)	34.0	1400	30	45

BA: Biliary atresia; INH: Idiopathic neonatal hepatitis; PFIC: Progressive familial intrahepatic cholestasis.

Table 3 Frequencies of selected variables for each diagnosed cause of infantile cholestasis *n* (%)

Variable		Frequency	BA	INH	Paucity of bile ducts	Choledochal cyst	Metabolic disease	Cirrhosis	PFIC and portal fibrosis
Sex	Female	28 (50.9)	20	3	1	1	2	0	1
	Male	27 (49.1)	12	2	5	1	4	2	1
Maturity	Preterm	15 (27.3)	8	3	1	0	1	1	1
	Term	40 (72.7)	24	2	5	2	5	1	1
Birth weight	Low	15 (27.3)	7	3	1	1	0	2	1
	Normal	40 (72.7)	25	2	5	1	6	0	1
Perinatal findings	Bacterial infection	5 (9.0)	3	0	0	0	1	0	1
	Congenital anomalies	10 (18.0)	4	1	1	2	0	2	0
	Parenteral nutrition	1 (2.0)	0	0	0	0	0	1	0
	Seizure	3 (5.5)	3	0	0	0	0	0	0
	Meconium stained amniotic fluid	4 (7.2)	2	0	2	0	0	0	0
	Fetomaternal hemorrhage	1 (2.0)	1	0	0	0	0	0	0
	Apnea	1 (2.0)	1	0	0	0	0	0	0
	Others	13 (23.0)	7	1	2	1	0	2	0
	Nil	31 (56.0)	18	4	3	0	5	0	1
Clinical findings	Dark urine	21 (38.0)	13	3	4	0	1	0	0
	Acholic stool	33 (60.0)	22	2	4	2	3	0	0
	Hepatomegaly	30 (54.5)	19	5	1	0	4	0	1
	Splenomegaly	17 (31.0)	12	2	0	0	2	0	1
	Clubbing	1 (2.0)	0	0	0	0	1	0	0
	Failure to thrive	14 (25.5)	9	1	1	0	2	1	0
	Ascites	8 (14.5)	4	1	1	0	1	0	1
	Jaundice	55 (100)	32	5	6	2	6	2	2
	Pruritus	3 (5.5)	1	0	0	0	1	0	1
Jaundice temporal course	Progressive	33 (60.0)	17	5	2	2	4	1	2
	Intermittent	4 (7.2)	3	0	1	0	0	0	0
	Continuous	6 (10.9)	3	0	1	0	1	1	0

BA: Biliary atresia; INH: Idiopathic neonatal hepatitis; PFIC: Progressive familial intrahepatic cholestasis.

surgical intervention[7,22], adversely affecting nutritional support, control of complications such as ascites, and cost. Accordingly, it has been recommended that infants presenting with acholic stools should be referred to a pediatric gastroenterologist for urgent evaluation to rule out BA[2,27,28]. Attempts to restore biliary flow, such as with the Kasai procedure, should be performed in BA cases before two months of age[7,29,30] in experienced centers to increase the chance for a successful surgery[8,31,32].

Coagulopathy, measured by prothrombin time (PT) and PTT, is a serious complication that may be present in infantile cholestasis[4,6,33]. Obstructed biliary flow may cause fat malabsorption, resulting in a deficiency in vitamin K, a fat soluble vitamin. If the symptomatic prolongation of the PT and PTT remains uncorrected after vitamin K administration, this may indicate a hepatocellular injury that is secondary to biliary obstruction, as occurs in patients with prolonged jaundice[25], rather than a vitamin K deficiency. In the current study, we found abnormal PTT was significantly more common in those diagnosed with cirrhosis after a liver biopsy.

There are a number of limitations in extrapolating the results of this study. Our case series is composed of a highly selective group of infants with progressive cholestasis who underwent liver biopsies at the Ali-Asghar Children's Hospital. As a result, an undefined number of cases with a self-limiting course or diagnosed by other investigations have not been included in this study, thereby underrepresented the frequency of benign cases. The results of this study are most relevant for cases of progres-

Table 4 Mean laboratory findings from each diagnosed cause of infantile cholestasis

Cause	Total bilirubin in mg/dL	Direct bilirubin in mg/dL	ALP in IU/L	AST in IU/L	ALT in IU/L	GGT in IU/L	PT in sec	PTT in sec	Hgb in g/dL	Platelet $\times 10^3/\mu L$
BA	14.4	8.0	1230	313	310	597[a]	14.0	38	9.7	390
INH	10.5	6.0	1343	287	113	-	16.0	44	10.0	406
Paucity of bile ducts	11.5	6.5	1475	223	250	310	18.0	37	11.0	410
PFIC	28.0	13.0	1455	65	57	44	12.0	39	9.5	700
Choledochal cyst	11.5	5.2	1667	212	170	-	13.0	33	8.5	300
Metabolic disease	13.8	6.7	843	205	114	66	13.0	34	9.5	355
Cirrhosis	11.6	5.3	1710	226	45	-	16.0	76[a]	12.0	200
Portal fibrosis	8.0	5.1	453	380	120	-	13.5	39	10.0	187

[a]$P < 0.05$. ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transpeptidase; Hgb: Hemoglobin; PT: Prothrombin time; PTT: Partial thromboplastin time.

sive conjugated hyperbilirubinemia that are candidates for liver biopsy. Two counterpart studies that mainly recruited cases from clinical diagnosis rather than liver biopsies, found INH, not BA, to be the most common cause of infantile cholestasis[12,13]. While some familial INH cases may represent unrecognized underlying inborn errors, specifically defects in synthesis or transport of bile acids, the sporadic INH is usually transient and has a rather favorable outcome[14,34-36], which is in stark contrast to the serious complications caused by BA.

In summary, we found that BA was the most common cause of infantile cholestasis as determined by liver biopsies. Additionally, there was a significant delay from the recognition of jaundice to the time of liver biopsy, averaging approximately two months. Considering the rapid progression of BA to cirrhosis along with the potentially curative role of early surgical intervention, an emphasis should be placed on obtaining prompt diagnostic testing in all infants presenting with conjugated hyperbilirubinemia that lasts beyond the first two weeks of life.

COMMENTS

Background

The prevalence of individual causes of infantile cholestasis (IC) has changed in recent decades, shifting from idiopathic neonate hepatitis as the most common cause, to more clearly defined disorders such as progressive familial intrahepatic cholestasis and bile acid synthetic defects. However, biliary atresia (BA) remains an important contributor to IC.

Research frontiers

There is very little data on the causes of prolonged cholestatic jaundice during infancy in developing countries. The data that are available are mainly derived from clinical findings that are not necessarily pathologically based. A known cause of IC is BA, a condition requiring surgical intervention to avoid permanent liver damage. Therefore, it is crucial to identify when a liver biopsy is needed for a timely diagnosis to occur.

Innovations and breakthroughs

This study examined the diagnoses of a selected population of IC patients who underwent liver biopsies. The frequencies of final diagnoses, as based on histopathology, and their time courses were evaluated. The results reveal that BA is in fact the most frequent diagnosis made from biopsied cases, with an unfavorable delay of 87.5 d after birth, on average.

Applications

The frequency data reported in this study provide a framework for physicians to reference regarding the timely diagnosis for infants with progressive conjugated hyperbilirubinemia. This study also highlights the need to consider early liver biopsies in cases of IC to avoid irreversible hepatic damage from causative conditions that are frequently diagnosed.

Terminology

Infantile cholestasis is a liver condition in neonatal infants describing direct hyperbilirubinemia that persists or appears after 14 d of life. Direct hyperbilirubinemia refers to conjugated serum bilirubin of more than 1 mg/dL when the total bilirubin is less than 5 mg/dL or if conjugated bilirubin accounts for greater than 20% of total serum bilirubin in cases of more severe hyperbilirubinemia. Biliary atresia is a potentially life-threatening blockage of bile ducts that occurs in infants.

Peer review

The authors examined the frequencies of diagnoses made from liver biopsies of infantile cases of cholestasis. The analysis shows that a very serious and potentially life-threatening condition is the most common cause of pediatric liver disease in the population examined. This identification suggests that liver biopsies should be more routinely considered in cases of IC, as early intervention is crucial for successful recovery. The authors document a significant delay between the appearance of conjugated jaundice in pediatric patients and the liver biopsy, which is required for an accurate diagnosis. Therefore, it is recommended that neonatologists and pediatricians conduct prompt diagnostic workups in all infants presenting with conjugated jaundice beyond the second week of life.

REFERENCES

1 **American Academy of Pediatrics Subcommittee on Hyperbilirubinemia.** Management of hyperbilirubinemia in the newborn infant 35 or more weeks of gestation. *Pediatrics* 2004; **114**: 297-316 [PMID: 15231951 DOI: 10.1542/peds.114.1.297]

2 **De Bruyne R**, Van Biervliet S, Vande Velde S, Van Winckel M. Clinical practice: neonatal cholestasis. *Eur J Pediatr* 2011; **170**: 279-284 [PMID: 21249394 DOI: 10.1007/s00431-010-1363-8]

3 **Emerick KM**, Whitington PF. Neonatal liver disease. *Pediatr Ann* 2006; **35**: 280-286 [PMID: 16637557]

4 **Venigalla S**, Gourley GR. Neonatal cholestasis. *Semin Perinatol* 2004; **28**: 348-355 [PMID: 15686266 DOI: 10.1053/j.semperi.2004.09.008]

5 **Moyer V**, Freese DK, Whitington PF, Olson AD, Brewer F, Colletti RB, Heyman MB. Guideline for the evaluation of cholestatic jaundice in infants: recommendations of the North American Society for Pediatric Gastroenterology, Hepatology and Nutrition. *J Pediatr Gastroenterol Nutr* 2004; **39**: 115-128 [PMID: 15269615 DOI: 10.1097/00005176-200408000-00001]

6 **Walker WA**, Goulet O, Kleinman RE, Sanderson IR, Sherman P, Shneider B. Pediatric Gastrointestinal Disease: Pathophysiology, Diagnosis, Management. 5th edition. Hamilton, Ontario: BC Decker Inc., 2008

7 **Schreiber RA**, Kleinman RE. Biliary atresia. *J Pediatr Gastroenterol Nutr* 2002; **35** Suppl 1: S11-S16 [PMID: 12151815]

8 **Lai MW**, Chang MH, Hsu SC, Hsu HC, Su CT, Kao CL, Lee CY. Differential diagnosis of extrahepatic biliary atresia from

neonatal hepatitis: a prospective study. *J Pediatr Gastroenterol Nutr* 1994; **18**: 121-127 [PMID: 8014758 DOI: 10.1097/00005176-199402000-00001]
9 Wyllie R, Hyams JS. Pediatric gastrointestinal disease, pathophysiology, diagnosis, management. 3rd edition. Philadelphia, PA: Saunders Elsevier, 2006: 869-883
10 **Nayak NC**, Vasdev N. Neonatal cholestasis syndrome: identifying the disease from liver biopsy. *Indian Pediatr* 2002; **39**: 421-425 [PMID: 12037270]
11 **Fallahi GH,** Farahmand F, NematKhorasani E. [Etiologic assessment of neonatal cholestasis: A six year study in children medical center, Tehran]. *TUMJ* 2007; **65**: 82-86
12 **Rafeey M**, Mahalehei M. Neonatal cholestasis. *Iran J Pediatr* 2005; **15**: 43-50
13 **Najafi M**, Khodadad A, Khatami Gh R, Farahmand F, Falahi Gh H, Motamed F. Prevalence of different etiology in neonatal cholestasis. *Iran J Pediatr* 2006; **16**: 289-294
14 **Mowat AP**, Psacharopoulos H.D, Williams R. Extrahepatic biliary atresia versus neonatal hepatitis: review of 137 prospectively investigated infants. *Arch Dis Child* 1976; **51**: 763-770 [PMID: 1087549]
15 **Sokol RJ**, Mack C, Narkewicz MR, Karrer FM. Pathogenesis and outcome of biliary atresia: current concepts. *J Pediatr Gastroenterol Nutr* 2003; **37**: 4-21 [PMID: 12827000 DOI: 10.1097/00005176-200307000-00003]
16 **Bezerra JA**, Balistreri WF. Cholestatic syndromes of infancy and childhood. *Semin Gastrointest Dis* 2001; **12**: 54-65 [PMID: 11352121]
17 **Sokol RJ**, Mack C. Etiopathogenesis of biliary atresia. *Semin Liver Dis* 2001; **21**: 517-524 [PMID: 11745039 DOI: 10.1055/s-2001-19032]
18 **McKiernan PJ**. Neonatal cholestasis. *Semin Neonatol* 2002; **7**: 153-165 [PMID: 12208100 DOI: 10.1053/siny.2002.0103]
19 **Lee WS**, Chai PF. Clinical features differentiating biliary atresia from other causes of neonatal cholestasis. *Ann Acad Med Singapore* 2010; **39**: 648-654 [PMID: 20838708]
20 **Finegold MJ**. Common diagnostic problems in pediatric liver pathology. *Clin Liver Dis* 2002; **6**: 421-454 [PMID: 12122864 DOI: 10.1016/S1089-3261(02)00014-4]
21 **Petersen C**, Harder D, Abola Z, Alberti D, Becker T, Chardot C, Davenport M, Deutschmann A, Khelif K, Kobayashi H, Kvist N, Leonhardt J, Melter M, Pakarinen M, Pawlowska J, Petersons A, Pfister ED, Rygl M, Schreiber R, Sokol R, Ure B, Veiga C, Verkade H, Wildhaber B, Yerushalmi B, Kelly D. European biliary atresia registries: summary of a symposium. *Eur J Pediatr Surg* 2008; **18**: 111-116 [PMID: 18437656 DOI: 10.1055/s-2008-1038479]
22 **Yachha SK**, Mohindra S. Neonatal cholestasis syndrome: Indian scene. *Indian J Pediatr* 1999; **66**: S94-S96 [PMID: 11132478]
23 **Chardot C**. Treatment for biliary atresia in 2003. *J Pediatr Gastroenterol Nutr* 2003; **37**: 407-408 [PMID: 14508208 DOI: 10.1097/00005176-200310000-00002]
24 **Pashankar D**, Schreiber RA. Neonatal cholestasis: a red alert for the jaundiced newborn. *Can J Gastroenterol* 2000; **14** Suppl D: 67D-72D [PMID: 11110615]
25 **Kliegman RM**, Stanton BF, Schor NF, Behrman RE, Geme JW. Nelson textbook of pediatrics. 19th edition. Philadelphia: Elsevier Saunders, 2011: 1381-1388
26 **Mieli-Vergani G**, Howard ER, Portman B, Mowat AP. Late referral for biliary atresia--missed opportunities for effective surgery. *Lancet* 1989; **1**: 421-423 [PMID: 2563796]
27 **Gilmour SM**. Prolonged neonatal jaundice: When to worry and what to do. *Paediatr Child Health* 2004; **9**: 700-704 [PMID: 19688078]
28 Sucky F, Sokol RJ, Balistreri WF. Liver disease in children. 3rd edition. Oxford city: Cambridge University Press, 2007: 179-190
29 **Sokol RJ**. Swiss outcomes in biliary atresia: are there lessons to be learned? *J Pediatr Gastroenterol Nutr* 2008; **46**: 238-240 [PMID: 18376239 DOI: 10.1097/MPG.0b013e318163357a]
30 **Volpert D**, White F, Finegold MJ, Molleston J, Debaun M, Perlmutter DH. Outcome of early hepatic portoenterostomy for biliary atresia. *J Pediatr Gastroenterol Nutr* 2001; **32**: 265-269 [PMID: 11345173 DOI: 10.1097/00005176-200103000-00006]
31 **McKiernan PJ**, Baker AJ, Lloyd C, Mieli-Vergani G, Kelly DA. British paediatric surveillance unit study of biliary atresia: outcome at 13 years. *J Pediatr Gastroenterol Nutr* 2009; **48**: 78-81 [PMID: 19172128 DOI: 10.1097/MPG.0b013e31817d80de]
32 **Wildhaber BE**, Majno P, Mayr J, Zachariou Z, Hohlfeld J, Schwoebel M, Kistler W, Meuli M, Le Coultre C, Mentha G, Belli D, Chardot C. Biliary atresia: Swiss national study, 1994-2004. *J Pediatr Gastroenterol Nutr* 2008; **46**: 299-307 [PMID: 18376248 DOI: 10.1097/MPG.0b013e3181633562]
33 **van den Anker JN**, Sinaasappel M. Bleeding as presenting symptom of cholestasis. *J Perinatol* 1993; **13**: 322-324 [PMID: 8410391]
34 **Balistreri WF**, Bezerra JA, Jansen P, Karpen SJ, Shneider BL, Suchy FJ. Intrahepatic cholestasis: summary of an American Association for the Study of Liver Diseases single-topic conference. *Hepatology* 2005; **42**: 222-235 [PMID: 15898074 DOI: 10.1002/hep.20729]
35 **Chang MH**, Hsu HC, Lee CY, Wang TR, Kao CL. Neonatal hepatitis: a follow-up study. *J Pediatr Gastroenterol Nutr* 1987; **6**: 203-207 [PMID: 2826743 DOI: 10.1097/00005176-198703000-00006]
36 **Setchell KD**, Suchy FJ, Welsh MB, Zimmer-Nechemias L, Heubi J, Balistreri WF. Delta 4-3-oxosteroid 5 beta-reductase deficiency described in identical twins with neonatal hepatitis. A new inborn error in bile acid synthesis. *J Clin Invest* 1988; **82**: 2148-2157 [PMID: 3198770 DOI: 10.1172/JCI113837]

P- Reviewers: Luo GH, Ivanovski PI **S- Editor**: Qi Y
L- Editor: A **E- Editor**: Wu HL

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.1054

World J Gastroenterol 2014 January 28; 20(4): 1054-1060
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Intra-gastric triacetin alters upper gastrointestinal motility in conscious dogs

Kazumasa Oosaka, Masaaki Tokuda, Naohiro Furukawa

Kazumasa Oosaka, Research and Development Department, Fushimi Pharmaceutical Co., Ltd., Kagawa 7638605, Japan
Masaaki Tokuda, Department of Cell Physiology, Faculty of Medicine, Kagawa University, Kagawa 7610793, Japan
Naohiro Furukawa, Department of Clinical Nutrition, Faculty of Health Science and Technology, Kawasaki University of Medical Welfare, Kurashiki 7010193, Japan
Author contributions: Oosaka K and Furukawa N contributed equally in performing the research, analyzing the data, and writing the manuscript; and Tokuda M contributed to the design of the research project.
Correspondence to: Dr. Kazumasa Oosaka, Research and Development Department, Fushimi Pharmaceutical Co., Ltd., Nakatsu-cho 1676, Marugame, Kagawa 7638605, Japan. ohsaka@fushimi.co.jp
Telephone: +81-877-226272 Fax: +81-877-226246
Received: August 31, 2013 Revised: October 30, 2013
Accepted: December 5, 2013
Published online: January 28, 2014

Abstract

AIM: To examine the effect of intra-gastric triacetin on both upper gastrointestinal motility and proximal gastric tone in conscious dogs.

METHODS: Three beagle dogs under sedation were surgically implanted with gastrocutaneous fistula in the gastric body and force transducers in the gastric antrum and duodenum. Beginning at week-2 after insertion, the animals were either fasted for 24 h or fed a liquid meal 2-3 h before the experiment. With the animals fully conscious, a polyethylene bag was inserted into the proximal stomach through the gastrocutaneous fistula, followed by 15 min of air inflation (minimal distending pressure of +2 mmHg) and then 20 mL of a low-, mid- or high-concentration triacetin solution (0.5%, 1.0% and 2.0%) or warm water (vehicle control). The proximal stomach receptive volume and gastric antral and duodenal contractions were measured over 10 min. The experiment was repeated twice per week over several months, with each animal receiving at least one infusion of the various triacetin solutions and the vehicle at different times. Intergroup differences were assessed by ANOVA and Bonferroni-Dunn post-hoc testing.

RESULTS: Intra-gastric infusion of mid- and high-concentration triacetin induced an increase in the proximal stomach receptive volume, and the average increase induced by the high-concentration at 0-4 min after infusion was significantly greater than that induced by the vehicle control (62.4 ± 9.8 *vs* 18.4 ± 4.7, $P < 0.01$). The mid- and high-concentration triacetin also produced a temporary inhibition of the gastric antral contractions at 2 min after infusions; however, only the fasted group showed triacetin-induced antral contractile inhibition that was significantly greater than that in the vehicle control group ($P < 0.05$). In addition, only the fasted group showed a high-concentration triacetin-induced increase in duodenal contractions at 9-10 min that was significantly different from that in the vehicle control group ($P < 0.05$).

CONCLUSION: Intra-gastric infusion of 1.0%-2.0% triacetin delays gastric emptying by increasing proximal stomach receptive volume, temporarily inhibiting gastric antral contractions and facilitating duodenal contractions.

Key words: Gastrointestinal motility; Barostat; Proximal gastric relaxation; Short-chain triglycerides

Core tip: Intra-gastric infusion of short-chain triglycerides, such as triacetin, has been shown to delay gastric emptying in conscious dogs, but the influence on upper gastrointestinal motility is unknown. The current study examined time-dependent changes in motility following intra-gastric triacetin administration at various doses in

conscious dogs and evaluated the effects of a fasted *vs* fed state. Compared to infusion of water (vehicle) alone, the 1.0% and 2.0% triacetin doses induced a significant increase in the proximal stomach receptive volume, a temporary inhibition of gastric antral contractions, and an increase in the duodenal contractions in fasted dogs.

Oosaka K, Tokuda M, Furukawa N. Intra-gastric triacetin alters upper gastrointestinal motility in conscious dogs. *World J Gastroenterol* 2014; 20(4): 1054-1060 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/1054.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.1054

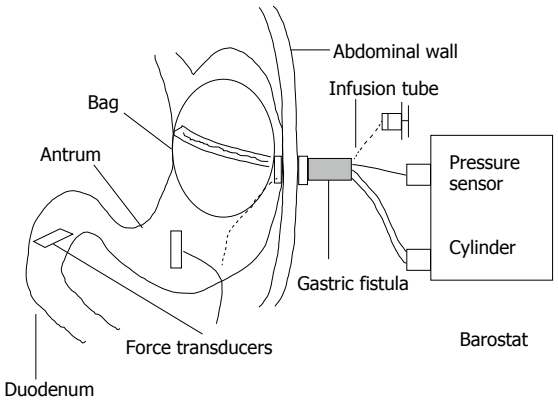


Figure 1 Schematic representation of canine experimental model.

INTRODUCTION

Triacetin is both the shortest-chain triglyceride (SCT), containing fatty acids with two carbons, and the only triglyceride that is soluble in water up to 6%. Its approval by the Food and Drug Administration as a safe human food ingredient has led to a series of studies examining its potential as a therapeutic agent for total parenteral nutrition[1-6]. While these studies have shown that triacetin can improve nitrogen balance[1] and protein metabolism[2], with a lack of toxicity[3], they have also shown minimal effects on mineral metabolism[4,5] due to the feature of water solubility. In an *in vivo* study by Lynch *et al*[6], wherein rats were fed diets containing triacetin to determine the effects on total adiposity, fat distribution and body composition, triacetin was shown to provide energy without accumulation in the body by decreasing adipocyte size. However, this field of research is relatively new and further investigations on the nutritive capacities and related mechanisms of triacetin are still in progress.

A number of other studies have examined the effects of long-chain triglycerides (LCTs) on gastrointestinal motility, demonstrating their effect of delaying gastric emptying and characterizing their feature of slow absorption. Specifically, it was shown that when digestive products of LCTs, such as mono- or diglycerides and long-chain fatty acids, are present in the duodenum and jejunum, the gastric emptying rate slows down[7,8], and that digestion and absorption of LCTs into the lymphatic system is dependent upon modification by bile salts. Moreover, Hunt *et al*[9] reported that gastric emptying is slower for 12- to 18-carbon fatty acids than for those composed of 2 to 10 carbons, suggesting that gastric emptying may be regulated in a manner that allows for optimal intestinal digestion and absorption of foodstuffs.

The processes of digestion and absorption of SCTs differ greatly from those of LCTs. SCTs do not require bile salts for digestion. Their passive diffusion from the gastrointestinal tract to the portal system has led to speculation that gastric emptying should not be delayed by SCTs. However, when triacetin was directly infused into the stomachs of conscious dogs, the gastric emptying rate was delayed remarkably[10]. Gastric emptying of a liquid is known to be facilitated by both the proximal gastric tone and antrum motility, both of which may also be influenced by the fasted/fed (postprandial) state. To determine whether triacetin can alter upper gastrointestinal motility during the observed delay of gastric emptying in conscious dogs a barostat and force transducers were applied to fasted and fed animals in the study described herein, and the effects of triacetin on upper gastrointestinal motility and the proximal gastric tone were comparatively analyzed.

MATERIALS AND METHODS

Animal preparation

All animal care and experimental procedures were approved by the Animal Research Committee of the Kawasaki Medical School (Japan) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals[11]. Three female beagle dogs (weight: 10-11 kg; age: 1-1.5 years) were anesthetized with medetomidine hydrochloride (20 μg/kg, subcutaneous) and pentobarbital sodium (25 mg/kg, intravenous). A midline laparotomy was performed, and a stainless steel gastrocutaneous fistula (inner diameter: 15 mm; length: 80 mm) was inserted into the middle corpus, with the placement ensuring exteriorization on the left side of the abdomen. Two force transducers were then sewn onto the wall of the gastric antrum and duodenum, following the direction of the circular muscle. The force transducer on the antrum was sutured at approximately 3 cm from the pyloric ring, while the force transducer on the duodenum was sutured at approximately 5 cm distal to the pyloric ring (Figure 1). The lead wires of the force transducers were positioned to ensure percutaneous exteriorization of the bilateral scapulae lower margins. The animals were allowed to recuperate for two weeks, with daily monitoring for procedure-related complications, before experimentation was initiated.

Experimental procedure

The fully conscious prepared animals were evaluated in either the fasting phase (after 24 h fasting) or in the post-

prandial phase (2-3 h after feeding of a high-calorie, high-protein liquid diet). For each phase, the experimental procedure was initiated by opening the gastrocutaneous fistula and aspirating any contents from the stomach; the mean aspirated volume from the postprandial stage was 29 ± 2.3 mL (n = 24), with a residual ratio of 11%-13%. A polyethylene bag (diameter: 12-13 cm; capacity: 0-1000 mL) fitted with a double-lumen polyvinyl tube was then inserted through the gastrocutaneous fistula into the proximal stomach and slowly inflated with 400-500 mL of air to ensure proper positioning. After complete deflation of the bag, a polyvinyl tube was introduced into the stomach through the gastrocutaneous fistula, to allow for infusion of a triacetin solution or vehicle control (water). The double-lumen tube and the infusion tube were fixed with adhesive vinyl tape. Thereafter, the conscious dogs were placed in a sling with their legs touching the ground so that they were supported upright without pressure on the abdomen. The double-lumen tube was then connected to a barostat (Isobar-3; G and J Electronics, Toronto, Canada) and the bag was gradually inflated from 2 to 5 mmHg in 1 mmHg stepwise 1 min increments. The minimal distending pressure (MDP) was determined by increasing the intra-gastric pressure to the point where volume variations were induced by respiratory motions. In this study, bag volumes of 30-100 mL were required for the respiratory motions to influence intra-gastric pressure in the dogs; therefore, the MDP was maintained at approximately 3 mmHg throughout the experiment.

Experimental design

This study used a barostat to examine the acute effects on fundic relaxation and gastric antral and duodenal contractions in conscious dogs in accordance with the methods of Furukawa *et al*[12]. Briefly, the intra-gastric bag was insufflated with air for 15 min, with the additional +2 mmHg bringing the MDP to 5 mmHg. After the initiation of insufflation for 5 min, 20 mL of pre-warmed (37 ℃) 0.5%, 1% or 2% triacetin or water were infused directly into the stomach over a period of 30 s through an infusion tube in the gastrocutaneous fistula. At the same time as the infusions, the force transducers were used to continuously measure the gastric antral and duodenal contractions.

For each animal, the experimental procedures were performed twice per week over a period of several months. Each animal received at least one infusion of each of the triacetin solutions as well vehicle before completion of the experimental course. The total numbers of fasting and postprandial phase experiments were nearly identical for all three dogs.

Statistical analysis

During the 15 min gastric distension performed at a MDP of +2 mmHg, the intra-gastric bag volume was calculated as the gastric tone over 1 min intervals. According to Furukawa *et al*[12], the mean values of three intra-gastric bag volumes at 3, 4 and 5 min after initiation of the gastric distension were designated as the basal volume before intra-gastric infusion of either the triacetin solutions or water. To evaluate the effect of triacetin on the proximal stomach receptive volume at a constant distending pressure, the mean values of the 1 min interval after the infusion were compared with the mean values of the basal volume. In addition, the differences between the basal volume and the volume after the infusion were calculated at 1 min intervals, with the triacetin data for the periods between 0-10 min compared to those of water. The effect of triacetin on contractility was evaluated by plotting the relative values of gastrointestinal contractility size that were obtained at every minute interval and calculating the area under the curve (AUC). As per the method described by Furukawa *et al*[12], the average of the 1 min intervals of AUC from 3 to 5 min after the onset of distension was used as the control value. The changes in relative magnitudes of the contractions from the control values after the infusion were then compared between the triacetin and water administrations. These results were expressed as mean ± SE The significance of intergroup differences was assessed by ANOVA with Bonferroni-Dunn post-hoc testing.

RESULTS

Effects of intra-gastric triacetin on proximal gastric tone

Fasting phase: A slight increase was observed in the intra-gastric bag volume immediately after the infusion of water, but was not significantly different from the pre-infusion volume (Figure 2A). Infusion of 0.5% triacetin did not produce a significant increase in the receptive volume, and only a slight distention of the intra-gastric bag was observed that was similar to that seen for the water infusion (data not shown). In contrast, infusion of 1.0% triacetin led to a rapid increase in the intra-gastric bag mean volume, from the mean basal volume of 129.3 ± 10.2 mL to a peak of 189.8 ± 14.5 mL at 1 min (n = 8). In addition, infusion of 2.0% triacetin led to a similar rapid increase, from the mean basal volume of 109.4 ± 7.2 mL to 179.1 ± 16.2 mL at 1 min (n = 7) and a peak of 181.2 ± 14.1 mL at 2 min (n = 7). These increases in the receptive volume induced by 1% and 2% triacetin infusions were significantly greater than the water infusion (Figure 2A).

Postprandial phase: In the postprandial phase, a slight increase in the receptive volume was observed that was similar to that seen during the fasting phase. Infusions of 1.0% and 2.0% triacetin led to rapid increases in the receptive volume, which were greater than those induced by the water infusion but the differences did not reach statistical significance (Figure 2B).

Time-related effects of intra-gastric triacetin on proximal gastric tone

Fasting phase: At 0-2 min after infusion of 1.0% triacetin, the increase in the receptive volume (56.5 ± 10.1,

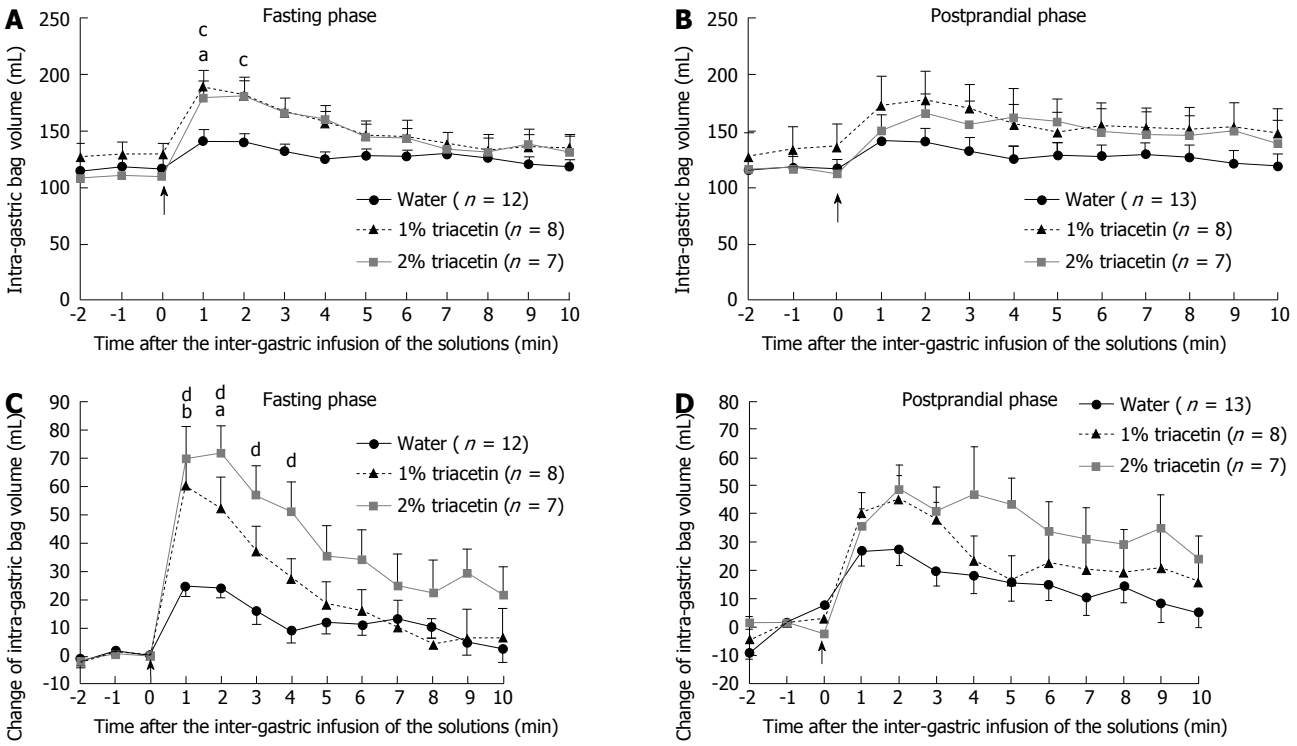


Figure 2 Effect of triacetin on the proximal gastric tone in fasting and postprandial phases. The receptive volume (representative of gastric tone) before and after infusion in fasted (A, C) and fed (B, D) animals is presented. Data are expressed as mean ± SE (A, B) $^{a}P < 0.05$ for 1% triacetin, $^{c}P < 0.05$ for 2% triacetin *vs* before infusion. (C, D) $^{a}P < 0.05$, $^{b}P < 0.01$ for 1% triacetin *vs* water; $^{c}P < 0.05$, $^{d}P < 0.01$ for 2% triacetin *vs* water. The point of infusion is indicated by an arrow.

n = 8) was significantly greater than that induced by the infusion of water alone (24.4 ± 3.7, n = 12). At 0-4 min after infusion of 2.0% triacetin, the increase in the receptive volume (62.4 ± 9.8, n = 7) was significantly greater than that induced by the infusion of water alone (18.4 ± 4.7, n = 12) (Figure 2C).

Postprandial phase: During the postprandial phase, the increases in the receptive volume induced by triacetin were also larger than those induced by water alone but the differences did not reach statistical significance (Figure 2D).

Effects of triacetin on gastric antral and duodenal contractions

Infusion of water alone produced no obvious changes in the gastric antral contractions, in neither the fasting and postprandial phases (Figure 3). In contrast, both 1.0% and 2.0% triacetin induced a temporary inhibition of the gastric antral contractions at 2 min after the infusion. Only the triacetin-induced inhibition in the fasting phase was significantly different from the contractions observed with the water infusion. However, after the 2 min time point, there was a tendency for facilitation of the gastric antral contractions only for the 2.0% triacetin in both the fasting and postprandial phases but the differences from the water-related contractions did not reach statistical significance.

At 1-4 min after the water infusion, a temporary facilitation of the duodenal contractions was observed in both the fasting and postprandial phases (Figure 4). For all time points thereafter, water induced only inhibition of the duodenal contractions. In contrast, infusion of 1.0% and 2.0% triacetin inhibited the duodenal contractions for nearly the entire 10-min measurement period in both phases, with the exception of 2.0% triacetin inducing a significant increase (*vs* water alone) in duodenal contractions at 9-10 min after the infusion.

DISCUSSION

The current study demonstrated that direct infusion of triacetin into the stomach of conscious dogs alters upper gastrointestinal motility. Proximal gastric volume was also increased by the infusion, which reflects stimulation of gastric relaxation. Finally, the triacetin was shown to inhibit gastric antral contractions but induce duodenal contractions.

Induction of gastric relaxation of the proximal stomach involves two mechanisms. First, gastric relaxation is stimulated by intra-gastric filling (possibly by expansion of the gastric wall[13]), regardless of the substance involved (solids or liquids). This process is referred to as gastric accommodation and is responsible for an increased volume tolerance without perception but may also contribute to maintaining satiation signals at acceptable volume loads. Second, physical (*e.g.*, osmotic pressure) and chemical (*e.g.*, H^+, lipids) parameters of the stomach and duodenum environments can enhance gastric relaxation *via* factors related to the nervous system or hormonal axes[14,15]. In the current study, triacetin did not appear to influence osmotic pressure, since the 0.5%, 1.0% and 2.0% triacetin was administered as hypotonic solutions. Moreover, the 20 mL volume that was infused

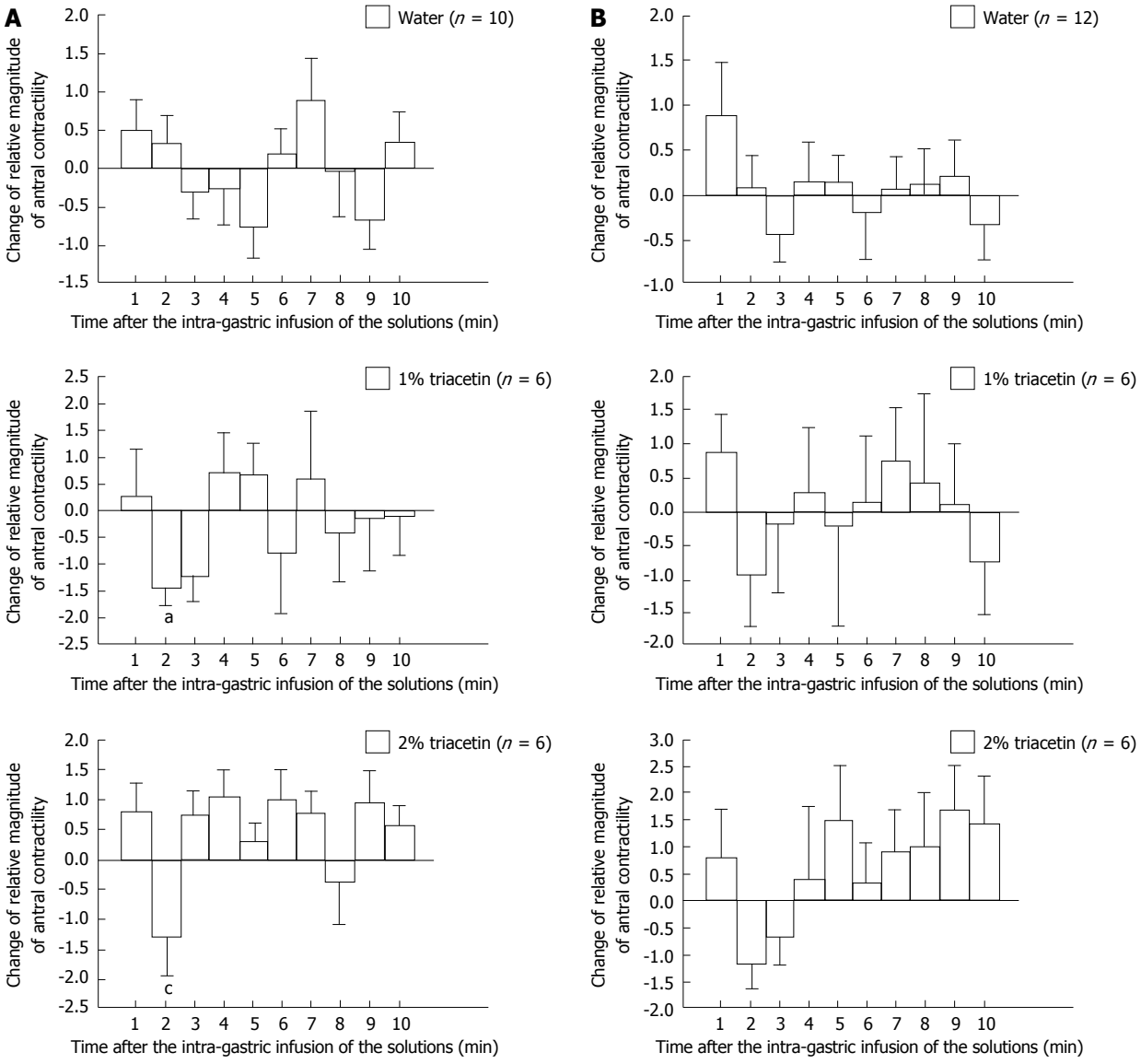


Figure 3 Effects of triacetin on gastric antral contractions in fasting and postprandial phases. The antral contractions recorded at every 1 min after infusion in (A) fasted and (B) fed animals are presented as the relative magnitudes of change from the average values at 3 min before infusion. Data are expressed as mean ± SE [a]*P* < 0.05 for 1% triacetin *vs* water, [c]*P* < 0.05 for 2% triacetin *vs* water.

into the stomach was not expected to cause expansion of the gastric wall. The triacetin infusion and subsequent absorption in the stomach and duodenum appeared to primarily affect upper gastrointestinal motility. However, the observation of duodenal facilitation occurring immediately after the infusion of triacetin suggests that the absorption-related process plays a smaller role than triacetin's chemical stimulation of the gastric and duodenal mucosa to causes the gastric relaxation.

Gastric antrum and duodenum contractions have distinctive mechanisms and patterns. For example, LCT-mediated effects on contractility are region specific. Particular LCTs capable of inhibiting antral contractility[16] have also been shown to facilitate stimulation of contractions in segments of the upper duodenum[17]. In the current study, the triacetin infusion first led to inhibition of gastric antral contractions, which was followed by an increase at later time points of the infusion. Moreover, the infusion appeared to facilitate duodenal contractility. Since there was only a short period of inhibition of the antral contractions, it remains unclear whether this inhibition was responsible for causing the delay of gastric emptying. However, such modulations of the gastric antrum and duodenum are expected to induce the closing of the pylorus, which may be the ultimate cause of the delayed gastric emptying. With regard to the observed diphasic alterations (*i.e.*, inhibition and facilitation) in the gastric antrum, the initial inhibition may be induced by stimulation of gastric antral mucosa, while the subsequent facilitation may be induced by stimulation of the duodenal mucosa.

LCTs are known to stimulate release of the hormone cholecystokinin (CCK), which acts to reduce proximal gastric tone[18] and inhibit gastric emptying[19] *via* activation of the CCK receptors on the vagal afferent nerves. In turn, the inhibitory vagal afferents in the brainstem are activated[20]. SCTs, on the other hand, produce very little

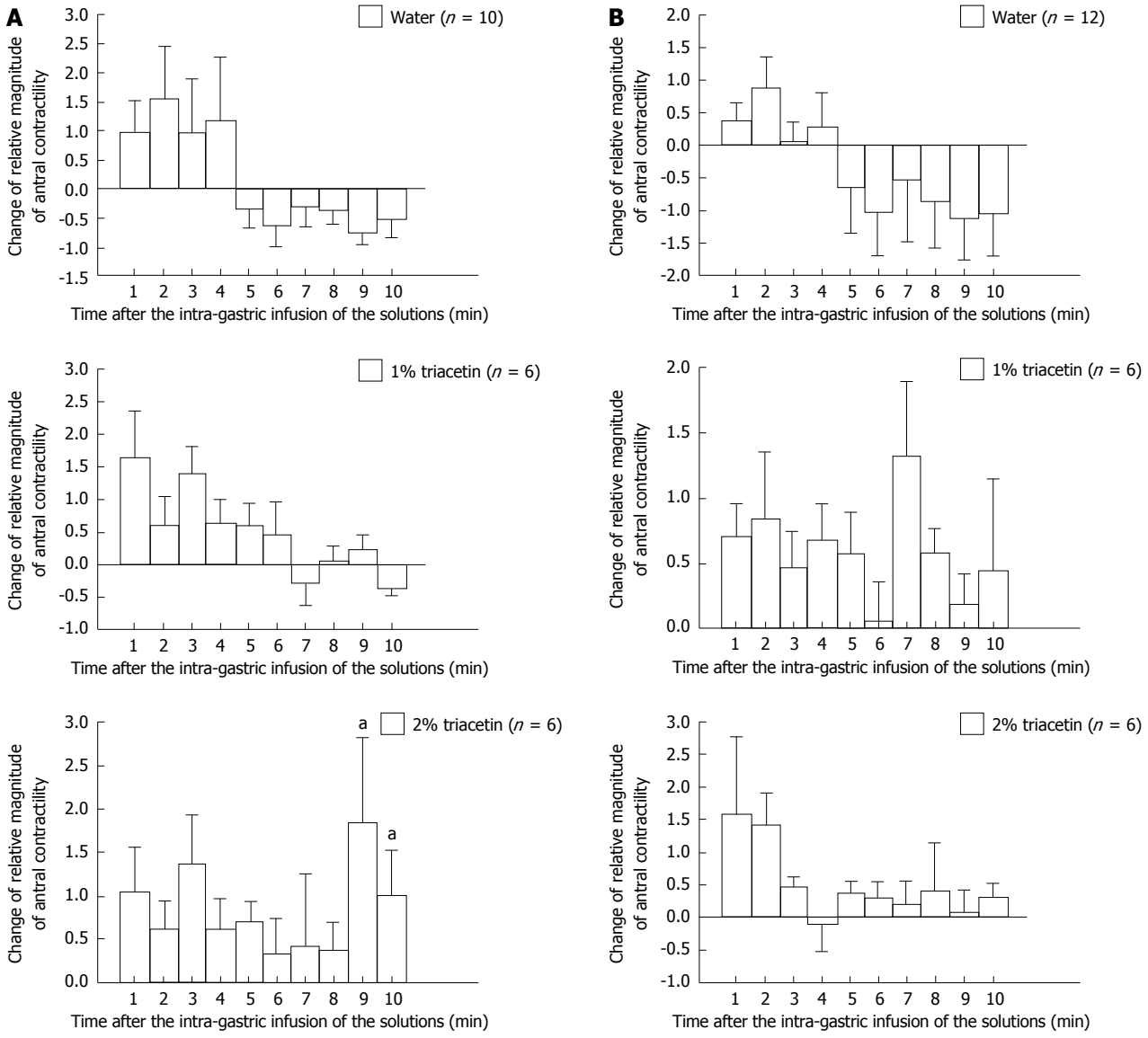


Figure 4 Effects of triacetin on duodenal contractions in fasting and postprandial phases. The duodenal contractions recorded at every 1 min after infusion in (A) fasted and (B) fed animals are presented as the relative magnitudes of change from the average values at 3 min before infusion. Data are expressed as mean ± SE [a]*P* < 0.05 for 2% triacetin *vs* water.

to no effects on CCK release[21]. Therefore, the mechanism of effect for triacetin, a SCT, may differ from that of the LCTs.

In the current study, the effects of triacetin related to fasting and fed states were also examined. While the results were largely similar for both the fasted and postprandial phases, statistically significant differences were only observed in the fasted animals. It is possible that the up-regulated hormonal factors that occur during the postprandial phase may have interfered with the triacetin, masking the effects.

In conclusion, our current results indicate that triacetin rapidly induces a temporary relaxation in the proximal stomach that is followed by contraction in both the gastric antrum and duodenum. This mechanism of triacetin may differ from that of LCTs, but further investigations are needed to confirm this distinction.

COMMENTS

Background

Gastric emptying is adjusted very precisely by gastrointestinal motility, so it is very important to examine the change of gastrointestinal motility after administrated the dietary ingredient. The authors reported preliminarily that an infusion of triacetin, which is expected as a new nutritional ingredient, into the stomach delayed the gastric emptying rate in conscious dogs. However, it is still unclear whether triacetin can change upper gastrointestinal motility during a delay of gastric emptying.

Research frontiers

Triacetin has been generally recognized by the Food and Drug Administration as being a safe human food ingredient. In the area of gastrointestinal motility with triacetin, the research hotspot is the change on the proximal gastric tone and the upper gastrointestinal motility.

Innovations and breakthroughs

In this study, to research the effect of triacetin on the gastric tone of the proximal stomach in conscious dogs, the authors used a polyethylene bag to measure the receptive volume of the proximal stomach at a constant pressure before and after the intra-gastric infusion of triacetin. Moreover, to determine the effect

of triacetin on the upper gastrointestinal contractile activities, the authors used force transducers to measure the gastric antral and duodenal contractions. The animals were either fasted for 24 h or fed a liquid meal 2-3 h before the experiment. Triacetin rapidly induces a temporary relaxation in the proximal stomach that is followed by contraction in both the gastric antrum and duodenum. The effect of triacetin was largely similar for both the fasted and postprandial phases, statistically significant differences were only observed in fasted animals.

Applications

Intra-gastric infusion of 1.0%-2.0% triacetin delays gastric emptying by increasing proximal stomach receptive volume, temporarily inhibiting gastric antral contractions and facilitating duodenal contractions.

Peer review

This manuscript reports novel information on the effect of short-chain triglycerides on gastric emptying in conscious dogs. The Authors demonstrate convincingly that Intra-gastric infusion of 1.0%-2.0% triacetin induced an increase of the receptive volume of the proximal stomach, and caused temporary inhibition of the gastric antral contractility, which are suggestive of a retardation of gastric emptying.

REFERENCES

1 **Bailey JW**, Barker RL, Karlstad MD. Total parenteral nutrition with short- and long-chain triglycerides: triacetin improves nitrogen balance in rats. *J Nutr* 1992; **122**: 1823-1829 [PMID: 1512631]

2 **Bailey JW**, Miles JM, Haymond MW. Effect of parenteral administration of short-chain triglycerides on leucine metabolism. *Am J Clin Nutr* 1993; **58**: 912-916 [PMID: 8249878]

3 **Bailey JW**, Haymond MW, Miles JM. Triacetin: a potential parenteral nutrient. *JPEN J Parenter Enteral Nutr* 1991; **15**: 32-36 [PMID: 1901105 DOI: 10.1177/0148607191050013 2]

4 **Bleiberg B**, Beers TR, Persson M, Miles JM. Metabolism of triacetin-derived acetate in dogs. *Am J Clin Nutr* 1993; **58**: 908-911 [PMID: 8249877]

5 **Bailey JW**, Heath H, Miles JM. Calcium, magnesium, and phosphorus metabolism in dogs given intravenous triacetin. *Am J Clin Nutr* 1989; **49**: 385-388 [PMID: 2492747]

6 **Lynch JW**, Bailey JW. Dietary intake of the short-chain triglyceride triacetin vs. long-chain triglycerides decreases adipocyte diameter and fat deposition in rats. *J Nutr* 1995; **125**: 1267-1273 [PMID: 7738686]

7 **Hunt JN**. Mechanisms and disorders of gastric emptying. *Annu Rev Med* 1983; **34**: 219-229 [PMID: 6344757 DOI: 10.1146/annurev.me.34.020183.001251]

8 **Houghton LA**, Mangnall YF, Read NW. Effect of incorporating fat into a liquid test meal on the relation between intragastric distribution and gastric emptying in human volunteers. *Gut* 1990; **31**: 1226-1229 [PMID: 2253903]

9 **Hunt JN**, Knox MT. A relation between the chain length of fatty acids and the slowing of gastric emptying. *J Physiol* 1968; **194**: 327-336 [PMID: 5639357]

10 **Oosaka K**. Effect of triacetin of gastric emptying. *J Smooth Muscle Res* (Jpn Section) 2005; **9**: 67-7811 [DOI: 10.1540/heikatsukinzashi1997.9.J67]

11 National Research Council (US) Committee for the update of the guide for the care and use of laboratory animals. Guide for the care and use of laboratory animals. 8th ed. Washington (DC): National Academies Press (US), 2011: 1-220

12 **Furukawa N**, Manabe N, Kase Y, Hattori T, Imamura H, Kusunoki H, Haruma K. Intragastric infusion of rikkunshito (kampo) induces proximal stomach relaxation in conscious dogs. *Auton Neurosci* 2013; **179**: 14-22 [PMID: 23907105 DOI: 10.1016/j.autneu.2013.06.003]

13 **Distrutti E**, Azpiroz F, Soldevilla A, Malagelada JR. Gastric wall tension determines perception of gastric distention. *Gastroenterology* 1999; **116**: 1035-1042 [PMID: 10220495 DOI: 10.1016/S0016-5085(99)7006-5]

14 **Jahnberg T**, Abrahamsson H, Jansson G, Martinson J. Gastric relaxatory response to feeding before and after vagotomy. *Scand J Gastroenterol* 1977; **12**: 225-228 [PMID: 847390]

15 **Barbera R**, Peracchi M, Brighenti F, Cesana B, Bianchi PA, Basilisco G. Sensations induced by medium and long chain triglycerides: role of gastric tone and hormones. *Gut* 2000; **46**: 32-36 [PMID: 10601051 DOI: 10.1136/gut.46.1.32]

16 **Heddle R**, Dent J, Read NW, Houghton LA, Toouli J, Horowitz M, Maddern GJ, Downton J. Antropyloroduodenal motor responses to intraduodenal lipid infusion in healthy volunteers. *Am J Physiol* 1988; **254**: G671-G679 [PMID: 3364568]

17 **Norman WW**. Gastric emptying. In: Leonard RJ, eds. Gastrointestinal Physiology, 6th edn. St. Louis: Mosby, Inc, 2001: 37-46

18 **Feinle C**, D'Amato M, Read NW. Cholecystokinin-A receptors modulate gastric sensory and motor responses to gastric distension and duodenal lipid. *Gastroenterology* 1996; **110**: 1379-1385 [PMID: 8613041 DOI: 10.1053/gast.1996.v110.pm8613041]

19 **Liddle RA**, Morita ET, Conrad CK, Williams JA. Regulation of gastric emptying in humans by cholecystokinin. *J Clin Invest* 1986; **77**: 992-996 [PMID: 3949984 DOI: 10.1172/JCI112401]

20 **Zarbin MA**, Wamsley JK, Innis RB, Kuhar MJ. Cholecystokinin receptors: presence and axonal flow in the rat vagus nerve. *Life Sci* 1981; **29**: 697-705 [PMID: 6168883 DOI: 10.1016/0024-3205(81)90022-9]

21 **Ledeboer M**, Masclee AA, Biemond I, Lamers CB. Effect of medium- and long-chain triglycerides on lower esophageal sphincter pressure: role of CCK. *Am J Physiol* 1998; **274**: G1160-G1165 [PMID: 9696717]

P- Reviewers: Francesco L, Izzo AA, Iwasaki Y **S- Editor**: Ma YJ
L- Editor: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.1061

World J Gastroenterol 2014 January 28; 20(4): 1061-1066
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Long-term pretreatment with proton pump inhibitor and *Helicobacter pylori* eradication rates

Seung Bae Yoon, Jae Myung Park, Jong-Yul Lee, Myong Ki Baeg, Chul-Hyun Lim, Jin Soo Kim, Yu Kyung Cho, In Seok Lee, Sang Woo Kim, Myung-Gyu Choi

Seung Bae Yoon, Jae Myung Park, Jong-Yul Lee, Myong Ki Baeg, Chul-Hyun Lim, Jin Soo Kim, Yu Kyung Cho, In Seok Lee, Sang Woo Kim, Myung-Gyu Choi, Division of Gastroenterology, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, Seoul 137-856, South Korea

Author contributions: Yoon SB and Park JM designed the study; Yoon SB and Park JM analyzed the results; Yoon SB, Park JM, Lee JY, Baeg MK, Lim CH, Kim JS, Cho YK, Lee IS, Kim SW and Choi MG collected the clinical data, wrote and edited the paper, and approved the final version.

Correspondence to: Jae Myung Park, MD, Division of Gastroenterology, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, Seoul St. Mary's Hospital, 505 Banpo-dong, Seocho-gu, Seoul 137-856, South Korea. parkjerry@catholic.ac.kr

Telephone: +82-2-22586023 Fax: +82-2-22582055

Received: August 12, 2013 Revised: September 20, 2013
Accepted: December 13, 2013
Published online: January 28, 2014

Abstract

AIM: To investigate whether proton pump inhibitor (PPI) pretreatment influences *Helicobacter pylori* eradication rate.

METHODS: We retrospectively reviewed *H. pylori*-infected patients who were treated with a standard triple regimen (PPI, amoxicillin 1 g, and clarithromycin 500 mg, all twice daily for 7 d). The diagnosis of *H. pylori* infection and its eradication was assessed with the rapid urease test, histological examination by silver staining, or the ^{13}C-urea breath test. We divided the patients into two groups: one received the standard eradication regimen without PPI pretreatment (Group A), and the other received PPI pretreatment (Group B). The patients in Group B were reclassified into three groups based on the duration of PPI pretreatment: Group B-Ⅰ (3-14 d), Group B-Ⅱ (15-55 d), and Group B-Ⅲ (≥ 56 d).

RESULTS: A total of 1090 patients were analyzed and the overall eradication rate was 80.9%. The cure rate in Group B (81.2%, 420/517) was not significantly different from that in Group A (79.2%, 454/573). The eradication rates in Group B-Ⅰ, B-Ⅱ and B-Ⅲ were 80.1% (117/146), 81.8% (224/274) and 81.4% (79/97), respectively.

CONCLUSION: PPI pretreatment did not affect *H. pylori* eradication rate, regardless of the medication period.

Key words: Proton pump inhibitor; *Helicobacter pylori*; Urea breath test; Antibiotics; Drug resistance

Core tip: Proton pump inhibitors (PPIs) are widely used for long periods. It is important to know whether long-term PPI pretreatment can influence *Helicobacter pylori* (*H. pylori*) eradication rates. There have been debates about the effect of PPI pretreatment on *H. pylori* eradication rate, although most previous studies have focused on the relatively short-term use of PPI. Our study investigated the impact of PPI pretreatment on *H. pylori* eradication rates based on different periods of treatment, including long-term pretreatment. Our data showed that PPI pretreatment did not affect *H. pylori* eradication rates, regardless of the medication period.

Yoon SB, Park JM, Lee JY, Baeg MK, Lim CH, Kim JS, Cho YK, Lee IS, Kim SW, Choi MG. Long-term pretreatment with proton pump inhibitor and *Helicobacter pylori* eradication rates. *World J Gastroenterol* 2014; 20(4): 1061-1066 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/1061.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.1061

INTRODUCTION

Several guidelines recommend standard triple therapy consisting of two antimicrobial agents, such as amoxicillin with clarithromycin or metronidazole, and a proton pump inhibitor (PPI) as the first choice treatment for *Helicobacter pylori* (*H. pylori*) infection[1-3]. The addition of a PPI to an antibiotic-containing regimen is known to boost the *H. pylori* eradication rate[4]. By increasing the intragastric pH, PPIs lower minimal inhibitory concentration (MIC) values and improve the chemical stability of antibacterial agents[5-7].

Although the inclusion of PPIs in the eradication regimen has been proven to be beneficial for curing *H. pylori* infection, it is still controversial whether PPI pretreatment influences the eradication rate. There was a recent study which showed that increasing the intragastric pH level by PPI pretreatment might improve the efficacy of *H. pylori* eradication[7]. Meanwhile, meta-analysis demonstrated that PPI pretreatment did not have any beneficial effect on *H. pylori* eradication[8]. Furthermore, some studies reported that PPI treatment before administering a single antibacterial agent, such as amoxicillin, decreases the eradication rate[9-11]. These findings have been explained by the fact that pretreatment induced the transition of *H. pylori* into coccoid dormant forms that are less vulnerable to the actions of antibiotics[12,13].

At present, endoscopic resection has been extensively applied to treat gastric neoplasms as a curative modality. This procedure inevitably results in a large iatrogenic ulcer, which subsequently poses the risk of gastric bleeding or perforation. To prevent these complications, PPIs are generally administered for > 4 wk[14,15]. However, recently there have been concerns raised about the possible adverse effects of long-term PPI treatment, including nutritional deficiencies, cardiovascular risk with PPI/clopidogrel co-prescriptions, and bone fractures[16,17]. Long-term PPI therapy should be used only in robust indications, and careful assessment of the risks and benefits is required.

In many cases, patients who received endoscopic resection with long-term PPI treatment need *H. pylori* eradication therapy because of its prophylactic effect on the development of metachronous gastric cancer[18-20]. From a clinical point of view, it is important to know whether long-term PPI pretreatment influences the *H. pylori* eradication rate. Previous studies have mostly focused on the effect of short-term PPI on *H. pylori* eradication, therefore, the effect of long-term PPI pretreatment is not yet clear. Our study was conducted to investigate the impact of PPI pretreatment on *H. pylori* eradication based on different periods of treatment duration, including long-term pretreatment.

MATERIALS AND METHODS

Patients

We retrospectively reviewed *H. pylori*-infected patients who were treated with a standard triple regimen from September 2009 to December 2011. The regimen consisted of PPI (lansoprazole 30 mg, esomeprazole 40 mg, or rabeprazole 20 mg), amoxicillin 1 g, and clarithromycin 500 mg, all twice daily for 7 d. Patients who completed the treatment and the assessment of eradication were enrolled in the study. Consumption of > 90% of the prescribed drugs was defined as good compliance and was accepted as the completion of treatment. The enrolled patients underwent upper gastrointestinal endoscopy before eradication treatment. The exclusion criteria were previous eradication therapy (n = 11), use of H_2 receptor antagonists or antibiotics within the past 4 wk (n = 58), being < 18 years (n = 3), and having an unknown history of recent medication (n = 35).

Assessment of H. pylori status

H. pylori infection was diagnosed according to one of the following tests: (1) rapid urease test (CLO test; Ballard Medical Products, Draper, UT, United States) by gastric mucosal biopsy from the body at the gastric angularis and greater curvature of the antrum; (2) histological examination by Warthin-Starry silver staining; and (3) ^{13}C-urea breath test (Helifinder; Medichems, Seoul, South Korea). The assessment of eradication was performed at least 4 wk after the completion of 1 wk of the standard regimen. The ^{13}C-urea breath test was generally used for the assessment of eradication, and rapid urease tests and histological examination were only used if repeat endoscopy was clinically indicated for other reasons.

Study design

We divided the patients into two groups: one received the standard eradication regimen without PPI pretreatment (Group A), and the other received the regimen with PPI pretreatment (Group B). PPI pretreatment in this study implied an intake of daily PPI (lansoprazole, rabeprazole, esomeprazole, or omeprazole) for ≥ 3 d before eradication therapy. Patients who received the eradication regimen within 3 d after the cessation of PPI pretreatment were enrolled in Group B, and those who received > 3 d were assigned to Group A. The rationale of these criteria was based on previous studies that demonstrated that the maximum effect of PPIs on the intragastric pH level occurred at least 3 d after the start of intake, and that the intragastric pH returned to the normal baseline level by 4 d after the cessation of PPI treatment[21,22]. Patients in Group B were reclassified into three groups based on the duration of PPI pretreatment: Group B-Ⅰ (3-14 d), Group B-Ⅱ (15-55 d), and Group B-Ⅲ (≥ 56 d). We also collected data from medical charts including demographic characteristics, diagnosis, types of PPI used in the pretreatment or eradication regimen, and eradication assessment methods. These factors might be potentially associated with eradication rate, thus, they were applied for adjustment.

Statistical analysis

Continuous data are presented as mean ± SD, and cat-

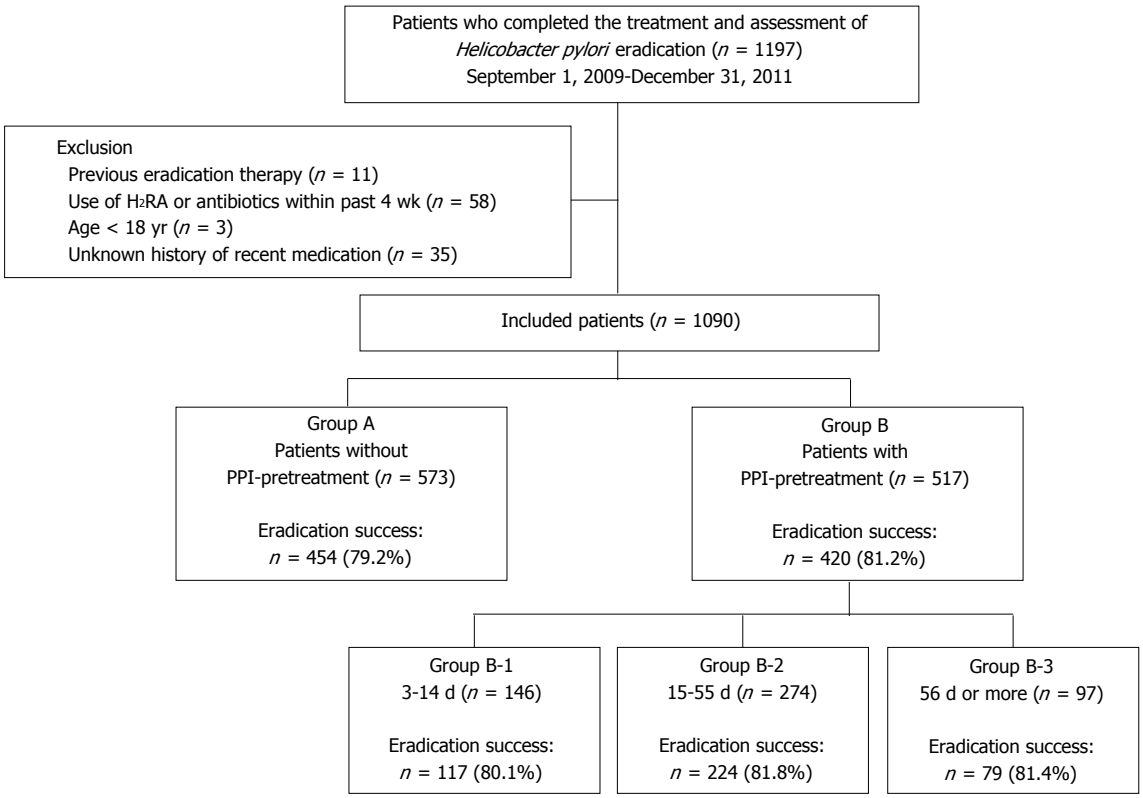


Figure 1 Flow of the study. A total of 1197 patients were investigated in the study period, and 573 patients in Group A [without proton pump inhibitors (PPI) pretreatment] and 517 in Group B (with PPI pretreatment) were included in the analysis. Patients in Group B were reclassified into three groups based on the duration of PPI pretreatment: Group B-1 (3-14 d), Group B-2 (15-55 d) and Group B-3 (≥ 56 d). H_2RA: H_2 receptor antagonist.

Table 1 Clinical characteristics and eradication rates of the two groups *n* (%)

		Group A without PPI pretreatment (*n* = 573)	Group B with PPI pretreatment (*n* = 517)	*P* value
Age	mean ± SD (yr)	51.8 ± 12.2	51.2 ± 13.4	0.453
Sex	Male	343 (59.9)	351 (67.9)	0.007
Diagnosis	Iatrogenic ulcer	31 (5.4)	107 (20.0)	< 0.001
	Peptic ulcer	438 (76.4)	395 (77.1)	
	Non-ulcer disease	104 (18.2)	15 (2.9)	
PPI in the eradication regimen	Lansoprazole 30 mg	439 (76.6)	410 (79.3)	0.021
	Esomeprazole 40 mg	92 (16.1)	56 (10.8)	
	Rabeprazole 20 mg	42 (7.3)	51 (9.9)	
Eradication assessment method	13C-urea breath test	525 (91.6)	408 (78.9)	< 0.001
	Rapid urease test	37 (6.5)	106 (20.5)	
	Histological examination	11 (1.9)	3 (0.6)	
Eradication rate		454 (79.2)	420 (81.2)	0.407

PPI: Proton pump inhibitors.

egorical data are presented as quantities and proportions. A χ^2 test or Fisher's exact test was used to analyze categorical data and the two-sample independent *t* test was used to analyze continuous data. Eradication rates were also investigated using adjusted logistic regression analysis. The analysis was performed using SAS software (SAS Institute, Cary, NC, United States), and statistical significance was accepted as $P < 0.05$.

RESULTS

After excluding 107 individuals from a total of 1197 patients enrolled in this study, we finally analyzed 1090 patients including 138 with iatrogenic ulcers caused by the endoscopic resection of gastric neoplasms. The retrospective assessment flow is presented in Figure 1. The mean age of the study group was 51.5 ± 12.8 years and 63.7% were male. The overall eradication rate was 80.2%.

Among the analyzed patients, 573 were enrolled in Group A and 517 in Group B. The baseline characteristics of the two groups are summarized in Table 1. The cure rate in Group B (81.2%, 420/517) was not significantly different from that in Group A (79.2%, 454/573, P = NS). In addition, the eradication rates were also not significantly different between Group A and B in any diagnostic subgroup; 87.1% (27/31) *vs* 76.6% (82/107) for iatrogenic ulcers, 77.6% (340/438) *vs* 82.3% (325/395) for peptic ulcer, and 83.7% (87/104) *vs* 86.7% (13/15) for non-ulcer disease (all P = NS). PPI pretreatment did not affect the eradication rate even after adjusting for age, sex, diagnosis, type of PPI in the eradication regimen, and eradication assessment methods (odds ratio, 1.14; 95% confidence interval, 0.83-1.58).

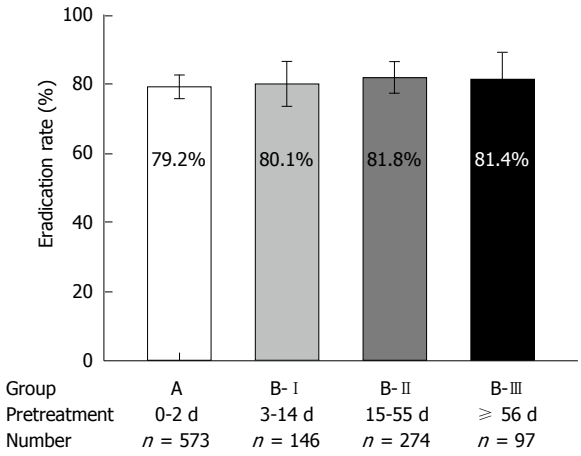


Figure 2 *Helicobacter pylori* eradication rates in different groups, bars indicate 95% confidence intervals. Eradication rates were not significantly different among Group A [without proton pump inhibitors (PPI) pretreatment], B-Ⅰ (PPI pretreatment for 3-14 d), B-Ⅱ (15-55 d), and B-Ⅲ (≥ 56 d) (P = 0.838).

The types of PPI for the pretreatment therapy in Group B-Ⅰ, B-Ⅱ and B-Ⅲ are shown in Table 2. The eradication rates in these three groups were 80.1% (117/146), 81.8% (224/274) and 81.4% (79/97), respectively. The eradication rates were not significantly different among these groups and Group A (P = NS; Figure 2).

DISCUSSION

The purpose of this study was to investigate the influence of PPI pretreatment on the rate of *H. pylori* eradication. There was no significant difference in the eradication rate between patients with and without pretreatment. PPI pretreatment did not affect the eradication rate, even after adjusting for various factors associated with eradication therapy. The present study also showed that the eradication rate was not affected by the duration of PPI pretreatment.

Generally, 80% and 85% cure rates based on intention-to-treat and per-protocol analysis, respectively, are regarded as the thresholds for acceptable results of *H. pylori* eradication[23]. In Korea, the eradication rates with 1 wk standard triple therapy have been reported to be similar[24]. In this regard, it is important to have a thorough knowledge of the factors that affect eradication rate, and we supposed that PPI pretreatment might be one of the possible factors. However, the present study showed that PPI pretreatment did not affect the eradication rate. Generally, patients with gastric neoplasms have lower gastric acid secretory function as compared with those with peptic ulcers, particularly duodenal ulcers. This possibly contributed to the similarity of the eradication rates between the study groups. Our findings were not different from the results of a previous meta-analysis[8].

Meanwhile, previous studies have mostly focused on the effect of the short-term use of PPIs. Therefore, the influence of long-term PPI pretreatment has not yet been elucidated. The effects of long-term PPI use are significant especially in the field of endoscopic resection therapy because the patients who receive this treatment need both

Table 2 Types of proton pump inhibitors used in the pretreatment in Group B *n* (%)

	Group B-Ⅰ 3-14 d (*n* = 146)	Group B-Ⅱ 15-55 d (*n* = 274)	Group B-Ⅲ ≥ 56 d (*n* = 97)	*P* value[1]
Lansoprazole	80 (54.8)	174 (63.5)	62 (63.9)	0.294
Rabeprazole	56 (38.4)	82 (29.9)	29 (29.9)	
Esomeprazole	8 (5.5)	17 (6.2)	4 (4.1)	
Omeprazole	2 (1.4)	1 (0.4)	2 (2.1)	

[1]Fisher's exact test was used.

the long-term use of PPIs and *H. pylori* eradication therapy. Although many studies have supported *H. pylori* eradication because of its prophylactic effect on the development of metachronous gastric cancer, these authors did not consider the effect of PPI pretreatment and the appropriate period for eradication therapy[18-20]. In the present study, we demonstrated that the eradication rates in long-term pretreatment groups were not different from those in the non-pretreatment and short-term pretreatment groups. This finding was observed in a recent study which showed that pretreatment with lansoprazole for 6-8 wk did not influence the eradication rate in peptic ulcer patients[25].

It seems that contrary to the negative effect of PPI treatment before the dual eradication regimen consisting of amoxicillin and PPI[9-11], PPI pretreatment does not affect the eradication rate in the clarithromycin-added triple regimen. Clarithromycin is known to be the most effective single agent against *H. pylori*[26], and has additive antibacterial activity when used with amoxicillin or PPIs[27]. By adding this powerful antibacterial agent to the regimen, the eradication rate does not seem to be affected by PPI pretreatment.

The main factor affecting the eradication rate of *H. pylori* might be its clarithromycin resistance as opposed to host factors[28,29]. Clarithromycin-resistant strains are barely eradicated with any dose of clarithromycin or PPI[30]. An *in vitro* study showed that the MIC values of clarithromycin for resistant strains remained high at various pH levels[31]. This finding implied that increases in intragastric pH by PPIs might not affect the eradication rates of clarithromycin-resistant strains. In Korea, the primary resistance rate of *H. pylori* to clarithromycin has been reported to be about 20%[32]; a level that is similar to the rates of eradication failure in our study. To rule out the possible confounders of clarithromycin resistance, we suggest that future studies are needed in patients with clarithromycin-sensitive strains.

CYP2C19 genotype status may also be associated with the eradication rate of *H. pylori*. A previous study reported that the eradication rate in extensive metabolizers was lower than that in poor metabolizers, and extensive metabolizers were successfully retreated with high doses of PPI[33]. We expected that PPI pretreatment might improve the eradication rate in extensive metabolizers because these people show a comparatively slower acid inhibitory effect after PPI use[34]. However, PPI pretreatment did not improve the overall eradication rate. We

could explain this result by the fact that the frequency of *CYP2C19* extensive metabolizers in East Asians is lower than that of Caucasians[35,36], and therefore the effect in extensive metabolizers might not be fully reflected in the outcomes of our study. For the maximized effect of PPI pretreatment, *CYP2C19* genotyping should be considered in future clinical research.

As a retrospective analysis, this study had several limitations. In particular, the enrolled patients received different types of PPI in the pretreatment and *H. pylori* eradication regimens, and underwent different methods for assessing the eradication. These inconsistencies for various factors were a weak point in our study. We tried to overcome these limitations by the enrollment of a large number of patients and by performing multivariate analysis. Additionally, any potential adverse effects of the PPIs were not investigated in our study. PPIs, however, were not prescribed for more than 6 mo in any patient (the longest, 168 d); and the adverse effects of long-term PPI treatment might be rare. Prospective and controlled studies are needed to confirm the present findings and complement the limitations of the study.

Guidelines recommend that the ^{13}C-urease breath test should be used for confirmation of eradication except in cases where repeat endoscopy is indicated[1,3]. In our study, the rapid urease test and histological examination were performed only in limited cases needing repeated endoscopy (14.4%). Although these tests have > 90% sensitivity and specificity in predicting *H. pylori* status after antibiotic treatment[37], the ^{13}C-urease breath test should be considered for confirming eradication.

In conclusion, PPI pretreatment did not affect the *H. pylori* eradication rates, regardless of the length of the medication period. Even after the long-term use of PPI treatment, *H. pylori* eradication can be attempted without worrying about its effect on eradication therapy.

ACKNOWLEDGMENTS

We acknowledge the financial support of the Catholic Medical Center Research Foundation in the program year of 2013.

COMMENTS

Background

After endoscopic resection of gastric neoplasms, acid-suppressive treatment with proton pump inhibitors (PPIs) is used for ≥ 1 mo, to prevent postprocedural complications and to enhance iatrogenic ulcer healing. In these patients, *Helicobacter pylori* (*H. pylori*) eradication decreases the recurrence of gastric neoplasms.

Research frontiers

PPIs in the eradication regimen have been beneficial for the cure of *H. pylori* infection. Some studies have reported that PPI treatment before the single antibacterial agent, such as amoxicillin, decreases the eradication rate, which can be explained by the fact that PPI pretreatment induces the transition of *H. pylori* into coccoid dormant forms that are less vulnerable to the actions of antibiotics. It is still controversial whether PPI pretreatment influences the eradication rate.

Innovations and breakthroughs

Most previous studies have assessed the effect of short-term use of PPIs on *H. pylori* eradication, therefore, the effect of long-term PPI pretreatment is not well known. Their study was conducted to investigate the influence of PPI pretreatment on *H. pylori* eradication, at different durations of treatment, including long-term pretreatment.

Applications

PPI pretreatment did not affect the *H. pylori* eradication rates, regardless of medication period. Even after long-term use of PPI treatment, *H. pylori* eradication can be tried without worrying about its effect on eradication therapy.

Peer review

The study aimed to answer the clinical question as to whether PPI pretreatment influences *H. pylori* eradication; especially when treatment was > 1 mo. This study had a large cohort size.

REFERENCES

1 **Chey WD**, Wong BC. American College of Gastroenterology guideline on the management of Helicobacter pylori infection. *Am J Gastroenterol* 2007; **102**: 1808-1825 [PMID: 17608775 DOI: 10.1111/j.1572-0241.2007.01393.x]

2 **Fock KM**, Katelaris P, Sugano K, Ang TL, Hunt R, Talley NJ, Lam SK, Xiao SD, Tan HJ, Wu CY, Jung HC, Hoang BH, Kachintorn U, Goh KL, Chiba T, Rani AA. Second Asia-Pacific Consensus Guidelines for Helicobacter pylori infection. *J Gastroenterol Hepatol* 2009; **24**: 1587-1600 [PMID: 19788600 DOI: 10.1111/j.1440-1746.2009.05982.x]

3 **Malfertheiner P**, Megraud F, O'Morain C, Bazzoli F, El-Omar E, Graham D, Hunt R, Rokkas T, Vakil N, Kuipers EJ. Current concepts in the management of Helicobacter pylori infection: the Maastricht III Consensus Report. *Gut* 2007; **56**: 772-781 [PMID: 17170018 DOI: 10.1136/gut.2006.101634]

4 **Lind T**, Mégraud F, Unge P, Bayerdörffer E, O'morain C, Spiller R, Veldhuyzen Van Zanten S, Bardhan KD, Hellblom M, Wrangstadh M, Zeijlon L, Cederberg C. The MACH2 study: role of omeprazole in eradication of Helicobacter pylori with 1-week triple therapies. *Gastroenterology* 1999; **116**: 248-253 [PMID: 9922303]

5 **Grayson ML**, Eliopoulos GM, Ferraro MJ, Moellering RC. Effect of varying pH on the susceptibility of Campylobacter pylori to antimicrobial agents. *Eur J Clin Microbiol Infect Dis* 1989; **8**: 888-889 [PMID: 2512133]

6 **Erah PO**, Goddard AF, Barrett DA, Shaw PN, Spiller RC. The stability of amoxycillin, clarithromycin and metronidazole in gastric juice: relevance to the treatment of Helicobacter pylori infection. *J Antimicrob Chemother* 1997; **39**: 5-12 [PMID: 9044021]

7 **Fan HY**, Wang J, Yan GC, Huo XH, Mu LJ, Chu JK, Niu WW, Duan ZY, Ma JC, Wang J, Wang ZY. Increasing gastric juice pH level prior to anti-Helicobacter pylori therapy may be beneficial to the healing of duodenal ulcers. *Exp Ther Med* 2013; **5**: 912-916 [PMID: 23408776 DOI: 10.3892/etm.2013.880]

8 **Janssen MJ**, Laheij RJ, de Boer WA, Jansen JB. Meta-analysis: the influence of pre-treatment with a proton pump inhibitor on Helicobacter pylori eradication. *Aliment Pharmacol Ther* 2005; **21**: 341-345 [PMID: 15709984 DOI: 10.1111/j.1365-2036.2005.02329.x]

9 **Labenz J**, Gyenes E, Rühl GH, Börsch G. Omeprazole plus amoxicillin: efficacy of various treatment regimens to eradicate Helicobacter pylori. *Am J Gastroenterol* 1993; **88**: 491-495 [PMID: 8470626]

10 **Labenz J**, Leverkus F, Börsch G. Omeprazole plus amoxicillin for cure of Helicobacter pylori infection. Factors influencing the treatment success. *Scand J Gastroenterol* 1994; **29**: 1070-1075 [PMID: 7886394]

11 **Bayerdörffer E**, Miehlke S, Mannes GA, Sommer A, Höchter W, Weingart J, Heldwein W, Klann H, Simon T, Schmitt W. Double-blind trial of omeprazole and amoxicillin to cure Helicobacter pylori infection in patients with duodenal ulcers. *Gastroenterology* 1995; **108**: 1412-1417 [PMID: 7729633]

12 **Nakao M**, Malfertheiner P. Growth inhibitory and bactericidal activities of lansoprazole compared with those of omeprazole and pantoprazole against Helicobacter pylori. *Helicobacter* 1998; **3**: 21-27 [PMID: 9546114]

13 **Goldstein NS**. Chronic inactive gastritis and coccoid Helicobacter pylori in patients treated for gastroesophageal re-

flux disease or with H pylori eradication therapy. *Am J Clin Pathol* 2002; **118**: 719-726 [PMID: 12428792 DOI: 10.1309/lj4d-e2lx-7umr-ymth]
14 **Uedo N**, Takeuchi Y, Yamada T, Ishihara R, Ogiyama H, Yamamoto S, Kato M, Tatsumi K, Masuda E, Tamai C, Yamamoto S, Higashino K, Iishi H, Tatsuta M. Effect of a proton pump inhibitor or an H2-receptor antagonist on prevention of bleeding from ulcer after endoscopic submucosal dissection of early gastric cancer: a prospective randomized controlled trial. *Am J Gastroenterol* 2007; **102**: 1610-1616 [PMID: 17403076 DOI: 10.1111/j.1572-0241.2007.01197.x]
15 **Yang Z**, Wu Q, Liu Z, Wu K, Fan D. Proton pump inhibitors versus histamine-2-receptor antagonists for the management of iatrogenic gastric ulcer after endoscopic mucosal resection or endoscopic submucosal dissection: a meta-analysis of randomized trials. *Digestion* 2011; **84**: 315-320 [PMID: 22075541 DOI: 10.1159/000331138]
16 **Abraham NS**. Proton pump inhibitors: potential adverse effects. *Curr Opin Gastroenterol* 2012; **28**: 615-620 [PMID: 23010681 DOI: 10.1097/MOG.0b013e328358d5b9]
17 **Chen J**, Yuan YC, Leontiadis GI, Howden CW. Recent safety concerns with proton pump inhibitors. *J Clin Gastroenterol* 2012; **46**: 93-114 [PMID: 22227731 DOI: 10.1097/MCG.0b013e3182333820]
18 **Fukase K**, Kato M, Kikuchi S, Inoue K, Uemura N, Okamoto S, Terao S, Amagai K, Hayashi S, Asaka M. Effect of eradication of Helicobacter pylori on incidence of metachronous gastric carcinoma after endoscopic resection of early gastric cancer: an open-label, randomised controlled trial. *Lancet* 2008; **372**: 392-397 [PMID: 18675689 DOI: 10.1016/s0140-6736(08)61159-9]
19 **Nakagawa S**, Asaka M, Kato M, Nakamura K, Kato C. Helicobacter pylori eradication and metachronous gastric cancer after endoscopic mucosal resection of early gastric cancer. Aliment *Pharmacol Ther* 2006; **24** Suppl 4: 214-218 [DOI: 10.1111/j.1365-2036.2006.00048.x]
20 **Uemura N**, Mukai T, Okamoto S, Yamaguchi S, Mashiba H, Taniyama K, Sasaki N, Haruma K, Sumii K, Kajiyama G. Effect of Helicobacter pylori eradication on subsequent development of cancer after endoscopic resection of early gastric cancer. *Cancer Epidemiol Biomarkers Prev* 1997; **6**: 639-642 [PMID: 9264278]
21 **Bell NJ**, Hunt RH. Time to maximum effect of lansoprazole on gastric pH in normal male volunteers. *Aliment Pharmacol Ther* 1996; **10**: 897-904 [PMID: 8971286]
22 **Bell N**, Karol MD, Sachs G, Greski-Rose P, Jennings DE, Hunt RH. Duration of effect of lansoprazole on gastric pH and acid secretion in normal male volunteers. *Aliment Pharmacol Ther* 2001; **15**: 105-113 [PMID: 11136283]
23 **Graham DY**, Lu H, Yamaoka Y. A report card to grade Helicobacter pylori therapy. *Helicobacter* 2007; **12**: 275-278 [PMID: 17669098 DOI: 10.1111/j.1523-5378.2007.00518.x]
24 **Kim N**, Park SH, Seo GS, Lee SW, Kim JW, Lee KJ, Shin WC, Kim TN, Park MI, Park JJ, Hong SJ, Shim KN, Kim SW, Shin YW, Chang YW, Chun HJ, Lee OJ, Jeon WJ, Park CG, Cho CM, Park CH, Won SY, Lee GH, Park KS, Shin JE, Kim HU, Park JY, Chae HS, Song GA, Kim JG, Yoon BC, Seol S, Jung HC, Chung IS. Lafutidine versus lansoprazole in combination with clarithromycin and amoxicillin for one versus two weeks for Helicobacter pylori eradication in Korea. *Helicobacter* 2008; **13**: 542-549 [PMID: 19166420 DOI: 10.1111/j.1523-5378.2008.00648.x]
25 **Inoue M**, Okada H, Hori S, Kawahara Y, Kawano S, Takenaka R, Toyokawa T, Onishi Y, Shiratori Y, Yamamoto K. Does pretreatment with lansoprazole influence Helicobacter pylori eradication rate and quality of life? *Digestion* 2010; **81**: 218-222 [PMID: 20090337 DOI: 10.1159/000260416]
26 **Peterson WL**, Graham DY, Marshall B, Blaser MJ, Genta RM, Klein PD, Stratton CW, Drnec J, Prokocimer P, Siepman N. Clarithromycin as monotherapy for eradication of Helicobacter pylori: a randomized, double-blind trial. *Am J Gastroenterol* 1993; **88**: 1860-1864 [PMID: 8237933]
27 **Cederbrant G**, Kahlmeter G, Schalén C, Kamme C. Additive effect of clarithromycin combined with 14-hydroxy clarithromycin, erythromycin, amoxycillin, metronidazole or omeprazole against Helicobacter pylori. *J Antimicrob Chemother* 1994; **34**: 1025-1029 [PMID: 7730216 DOI: 10.1093/jac/34.6.1025]
28 **Broutet N**, Tchamgoué S, Pereira E, Lamouliatte H, Salamon R, Mégraud F. Risk factors for failure of Helicobacter pylori therapy--results of an individual data analysis of 2751 patients. *Aliment Pharmacol Ther* 2003; **17**: 99-109 [PMID: 12492738 DOI: 10.1046/j.1365-2036.2003.01396.x]
29 **Yakoob J**, Jafri W, Abbas Z, Abid S, Naz S, Khan R, Khalid A. Risk factors associated with Helicobacter pylori infection treatment failure in a high prevalence area. *Epidemiol Infect* 2011; **139**: 581-590 [PMID: 20525411 DOI: 10.1017/S0950268810001226]
30 **Murakami K**, Sato R, Okimoto T, Nasu M, Fujioka T, Kodama M, Kagawa J, Sato S, Abe H, Arita T. Eradication rates of clarithromycin-resistant Helicobacter pylori using either rabeprazole or lansoprazole plus amoxicillin and clarithromycin. *Aliment Pharmacol Ther* 2002; **16**: 1933-1938 [PMID: 12390102 DOI: 10.1046/j.1365-2036.2002.01368.x]
31 **Lascols C**, Bryskier A, Soussy CJ, Tanković J. Effect of pH on the susceptibility of Helicobacter pylori to the ketolide telithromycin (HMR 3647) and clarithromycin. *J Antimicrob Chemother* 2001; **48**: 738-740 [PMID: 11679568 DOI: 10.1093/jac/48.5.738]
32 **Lee JW**, Kim N, Kim JM, Nam RH, Chang H, Kim JY, Shin CM, Park YS, Lee DH, Jung HC. Prevalence of primary and secondary antimicrobial resistance of Helicobacter pylori in Korea from 2003 through 2012. *Helicobacter* 2013; **18**: 206-214 [PMID: 23241101 DOI: 10.1111/hel.12031]
33 **Furuta T**, Shirai N, Takashima M, Xiao F, Hanai H, Sugimura H, Ohashi K, Ishizaki T, Kaneko E. Effect of genotypic differences in CYP2C19 on cure rates for Helicobacter pylori infection by triple therapy with a proton pump inhibitor, amoxicillin, and clarithromycin. *Clin Pharmacol Ther* 2001; **69**: 158-168 [PMID: 11240980 DOI: 10.1067/mcp.2001.113959]
34 **Saitoh T**, Fukushima Y, Otsuka H, Hirakawa J, Mori H, Asano T, Ishikawa T, Katsube T, Ogawa K, Ohkawa S. Effects of rabeprazole, lansoprazole and omeprazole on intragastric pH in CYP2C19 extensive metabolizers. *Aliment Pharmacol Ther* 2002; **16**: 1811-1817 [PMID: 12269976 DOI: 10.1046/j.1365-2036.2002.01348.x]
35 **Sim SC**, Risinger C, Dahl ML, Aklillu E, Christensen M, Bertilsson L, Ingelman-Sundberg M. A common novel CYP2C19 gene variant causes ultrarapid drug metabolism relevant for the drug response to proton pump inhibitors and antidepressants. *Clin Pharmacol Ther* 2006; **79**: 103-113 [PMID: 16413245 DOI: 10.1016/j.clpt.2005.10.002]
36 **Sugimoto K**, Uno T, Yamazaki H, Tateishi T. Limited frequency of the CYP2C19*17 allele and its minor role in a Japanese population. *Br J Clin Pharmacol* 2008; **65**: 437-439 [PMID: 18241287 DOI: 10.1111/j.1365-2125.2007.03057.x]
37 **Rollán A**, Giancaspero R, Arrese M, Figueroa C, Vollrath V, Schultz M, Duarte I, Vial P. Accuracy of invasive and noninvasive tests to diagnose Helicobacter pylori infection after antibiotic treatment. *Am J Gastroenterol* 1997; **92**: 1268-1274 [PMID: 9260787]

P- Reviewers: Clayton SB, Niu ZS, Shimatan T **S- Editor**: Qi Y
L- Editor: Kerr C **E- Editor**: Wang CH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.1067

World J Gastroenterol 2014 January 28; 20(4): 1067-1073
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Phase Ⅰ study of postoperative radiotherapy combined with capecitabine for gastric cancer

Xin Wang, Jing Jin, Ye-Xiong Li, Hua Ren, Hui Fang, Shu-Lian Wang, Yue-Ping Liu, Wei-Hu Wang, Zi-Hao Yu, Yong-Wen Song, Xin-Fan Liu

Xin Wang, Jing Jin, Ye-Xiong Li, Hua Ren, Hui Fang, Shu-Lian Wang, Yue-Ping Liu, Wei-Hu Wang, Zi-Hao Yu, Yong-Wen Song, Xin-Fan Liu, Department of Radiation Oncology, Cancer Hospital, National Cancer Center, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100021, China
Author contributions: Wang X, Jin J and Li YX designed the research; Wang X, Jin J, Li YX, Ren H, Fang H, Wang SL, Liu YP, Wang WH, Yu ZH, Song YW and Liu XF performed the research; Wang X collected and analyzed the data and wrote the paper; Jin J and Li YX modified the paper.
Supported by Beijing Hope Run Special Fund, No. LC2007A03
Correspondence to: Jing Jin, MD, Department of Radiation Oncology, Cancer Hospital, National Cancer Center, Chinese Academy of Medical Sciences and Peking Union Medical College, Panjiayuan Nanli 17, Chaoyang District, Beijing 100021, China. jingjin1025@163.com
Telephone: +86-10-87787658 Fax: +86-10-67706153
Received: September 27, 2013 Revised: November 14, 2013
Accepted: December 12, 2013
Published online: January 28, 2014

Abstract

AIM: To determine the maximum tolerated dose (MTD) and dose-limiting toxicity (DLT) of capecitabine combined with postoperative radiotherapy for gastric cancer.

METHODS: We enrolled patients with any T stage and node-positive gastroesophageal or gastric adenocarcinoma after complete resection with negative margins (R0) or microscopic (R1) or macroscopic (R2) resection. Intensity modulated radiotherapy (IMRT) using a five-to-seven-field, coplanar, sliding window technique was delivered to the tumor bed (T4b), anastomosis site, duodenal stump and regional lymph nodes (LNs) to a total dose of 45 Gy (1.8 Gy/fraction, 5 d/wk). Patients with R1 or R2 resection received 10.8 Gy as a boost. Capecitabine was administered twice daily on every radiotherapy treatment day in a dose-escalation schedule (mg/m^2) of 625 (level Ⅰ, n = 6), 700 (level Ⅱ, n = 6), 800 (level Ⅲ, n = 6), 900 (level Ⅳ, n = 0) and 1000 (level Ⅴ, n = 0). DLT was defined as grade 4 leukopenia or neutropenia, grade 3-4 thrombocytopenia or anemia and grade 3-4 non-hematological toxicity.

RESULTS: Between October 2007 and August 2009, 18 patients (12 men, 6 women; median age, 54 years) were enrolled in the study. The median number of positive LNs was 6, and total number of resected LNs was 19. Twelve patients underwent R0 resection (66.7%). Fifteen patients received adjuvant chemotherapy under the leucovorin, fluorouracil and oxaliplatin (FOLFOX4) regimen. Six patients each were enrolled at dose levels Ⅰ, Ⅱ and Ⅲ. Grade 1-3 leukopenia (16 patients, 88.9%), anorexia (15, 83.3%) and nausea (15, 83.3%) were the most common toxicities. Grade 3 anorexia/nausea and grade 4 vomiting occurred in one level-Ⅰ patient. Grade 3 anorexia and nausea occurred in one level-Ⅱ patient. One level-Ⅲ patient developed grade 4 neutropenia, while another developed grade 3 radiation esophagitis. No abnormal liver or renal function examinations were observed. Three patients did not finish chemoradiotherapy because of DLTs and two without DLTs received sequential boosts (total dose, 55.8 Gy).

CONCLUSION: The MTD of capecitabine was 800 mg/m^2 twice daily concurrent with IMRT for gastric cancer after surgery. The DLTs were anorexia/nausea, vomiting, neutropenia and radiation esophagitis.

Key words: Radiotherapy; Capecitabine; Gastric cancer; Maximum tolerated dose; Dose-limiting toxicity

Core tip: Postoperative chemoradiotherapy is a good option for patients with locally advanced, gastric cancer who have undergone R0 and D0-1 lymphadenectomy.

To avoid acute side effects and make the drug safer, a combination of the use of advanced techniques such as intensity modulated radiotherapy and mature chemotherapy regimens with capecitabine is highly recommended, especially in China which accounts for 40% of the world's gastric cancer patients. The aim of this single-institution, phase Ⅰ, clinical trial was to assess the feasibility and toxicity of a postoperative regimen involving dose escalation of capecitabine combined with IMRT for locally advanced gastric cancer.

Wang X, Jin J, Li YX, Ren H, Fang H, Wang SL, Liu YP, Wang WH, Yu ZH, Song YW, Liu XF. Phase Ⅰ study of postoperative radiotherapy combined with capecitabine for gastric cancer. *World J Gastroenterol* 2014; 20(4): 1067-1073 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/1067.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.1067

INTRODUCTION

Gastric cancer is the fourth most common cancer worldwide[1]. In China, gastric cancer has the third highest mortality rate among all cancers according to the latest Chinese Cancer Registry Annual Report[2]. A complete resection with negative margins (R0) remains the cornerstone of treatment for resectable gastric cancer. Nonetheless, less than 50% of patients will have an R0 resection of their primary tumor[3]. Therefore, long-term survival is poor, especially in patients with stage Ⅲ or Ⅳ gastric cancer. Based on the results of Intergroup study (INT) 0116, concurrent chemoradiotherapy (CRT) has been considered as the gold standard of treatment for patients with locally advanced gastric cancer who have undergone radical surgery (R0) and less than a D2 lymphadenectomy has been achieved[4]. In addition, postoperative radiotherapy (RT) with concurrent fluoropyrimidine is the main treatment for patients with residual disease after a microscopic (R1) or macroscopic (R2) resection, according to the National Comprehensive Cancer Network (NCCN) guidelines for gastric cancer[5,6].

The 5-fluorouracil (5FU) analog, capecitabine, has been widely used for gastric cancer treatment, either in chemotherapy regimens or in concurrent CRT. The results using capecitabine were found to be comparable to 5FU, and this drug carries a considerably safer side-effects profile and a more convenient oral route of administration[7-11].

According to INT 0116, the significant toxicities of capecitabine were of great concern when this drug was applied in routine clinical practice. However, conventional RT with anteroposterior opposing fields (AP-PA) to the upper abdomen contributed to the observed severe acute toxicities. The recently developed RT techniques, three dimensional conformal therapy (3DCRT) and intensity modulated radiotherapy (IMRT), are greatly superior to conventional RT because they spare more normal tissue and critical organs outside the radiation field[12-14]. Although some studies have evaluated conformal RT combined with 5FU infusion or conventional RT combined with capecitabine as postoperative treatments for gastric cancer, a new regimen of IMRT with concomitant capecitabine has not yet been investigated[9,15,16].

The aim of this single-institution, phase Ⅰ, clinical trial was to assess the feasibility and toxicity of a postoperative regimen involving the dose escalation of capecitabine combined with IMRT for locally advanced gastric cancer.

MATERIALS AND METHODS

Eligibility

The eligibility criteria included the following: (1) pathologically confirmed adenocarcinoma; (2) postoperative classification of anyTN + M0 according to the 7th edition of the American Joint Committee on Cancer TNM Classification[17]; (3) World Health Organization performance status of $\leqslant$ 1 and age $\leqslant$ 70 years; (4) no prior or concurrent malignancy (except non-melanoma skin cancers or in situ carcinoma of the cervix); (5) no history of abdominal radiation; and (6) hemoglobin level $\geqslant$ 10.0 g/L, leukocyte count $\geqslant$ 3.5×10^9/L, neutrophil count $\geqslant$ 1.5×10^9/L, platelet count $\geqslant$ 100×10^9/L and normal liver and kidney function.

The pretreatment workup consisted of physical examination, chest X-ray, abdominal and pelvic computed tomography (CT) scans (chest CT was included for proximal lesions), and a complete blood count and biochemical profile. Patients with heart disease that required medication, other severe comorbidities or psychiatric history which rendered them incapable of complying with the treatment regimen were excluded.

After being informed and having given their written consent, all patients who underwent R0/R1/R2 resection with pathologically proven, locally advanced gastric adenocarcinoma (anyTN + M0) were enrolled in this study. Patients who were administered any adjuvant chemotherapy before or after CRT were included in the study.

Radiotherapy

IMRT was selected because of its superior protocol design, which potentially reduces toxicities by reducing radiation exposure to adjacent normal structures. Patients were required to be fasted for 4 h before the CT simulation and take an oral positive contrast (300 mL) 30 min before CT simulation to make the small intestine visible. To decrease variability in distention due to gastric filling, a standard meal (300 mL of ready-to-eat canned porridge) was given to the patients 15 min before CT scanning and before each daily treatment. Intravenous administration of contrast was added for the IMRT; the patients were placed in a supine position with thermoplastic immobilization during IMRT with a 6-MV photon beam.

The gross tumor volume (GTV) encompassed either the visible, residual primary tumor or lymph nodes (LNs) based on the CT and/or positron-emission tomography-CT findings in patients who had undergone R2 resection

Table 1 Clinical target volume for elective nodal regions depending on the location of the primary gastric tumor[2]

Tumor location	CTV for elective nodal regions
Upper 1/3 or gastroesophageal junction	110, 1-3, 7, 9-11
Middle 1/3	1-3, 5-13, 14[1], 16a
Lower 1/3	3, 5-9, 11p, 12-13, 14[1], 16a

[1]No. 14 was included in the clinical target volume (CTV) only when the surface or parenchyma of the pancreas was involved by the tumor; [2]According to the guidelines issued by the Japanese Gastric Cancer Association. 110: Paraesophageal lymph nodes (LNs) in the lower thorax; 1: Right paracardial LNs; 2: Left paracardial LNs; 3: LNs along the lesser curvature; 5: Suprapyloric LNs; 6: Infrapyloric LNs; 7: LNs along the left gastric artery; 8: LNs along the common hepatic artery; 9: LNs around the celiac artery; 10: LNs at the splenic hilum; 11: LNs along the splenic artery (11p: LNs along the proximal splenic artery); 12: LNs in the hepatoduodenal ligament; 13: LNs on the posterior surface of the pancreatic head; 14: LNs along the root of the mesentery; 16a: LNs around the abdominal aorta (above the level of the inferior border of the left renal vein).

or the confirmable microscopic area in patients who had a R1 resection. The delineation of the clinical target volume (CTV) for each patient depended on the extension and location of the primary tumor and the guidelines for the involved LN region issued by the Japanese Gastric Cancer Association[18]. Generally, the CTV included the GTV (if present), anastomoses, duodenal stump, tumor bed (only for stage T4b, if present) and regional LNs (Table 1). The remnant stomach was not routinely included within the radiation field. The planning target volume (PTV) consisted of the CTV with a 0.5-0.7 cm margin in the radial direction and a 1 cm margin in the superior-inferior direction. For R1 or R2 resection, the GTV (if visible) plus a 0.5-0.7 cm three-dimensional extension formed a boost planning GTV. Dose constraints for organ at risk (OAR) were as follows: V30 (volume receiving a dose of 30 Gy or more) < 40% for the liver, V20 < 30% for both kidneys or a mean dose of < 20 Gy, V30 < 30% for the heart and the maximal dose for the spinal cord planning OAR volume was 40 Gy. With regards to the small bowel and colon, the maximal dose was less than the prescribed dose, and V50 < 10% was used for patients receiving an additional boost. An experienced physicist designed the IMRT plans using a five-to-seven-field, coplanar, sliding window technique on the Pinnacle system, version 3.0 (Figure 1).

A total irradiation dose of 45 Gy was delivered in daily 1.8-Gy fractions (5 d a week over 5 wk) to R0 patients, and a sequential 10.8-Gy boost was delivered in six fractions to either the visible residual tumor (R2 resection) or the confirmed microscopic area (R1 resection).

Chemotherapy

Capecitabine was administered twice daily (after breakfast and after dinner) from the beginning to the end of the duration of RT, in a dose-escalation schedule of 625 mg/m^2 (level Ⅰ), 700 mg/m^2 (level Ⅱ), 800 mg/m^2 (level Ⅲ), 900 mg/m^2 (level Ⅳ) and 1000 mg/m^2 (level Ⅴ).

If a dose-limiting toxicity (DLT) occurred in one of the first three patients, three additional patients were assigned to receive the same dose level. If none of the first three patients initially receiving a given dose level developed a DLT, or if only one of six patients had DLT, the dose was increased to the next level. If a second patient experienced a DLT at the same level, then the escalation was stopped, and the maximum tolerated dose was defined as the level at which the DLT occurred in this protocol.

Safety assessment

Adverse events were coded in accordance with the National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0. DLTs were defined as follows: grade 4 leukopenia or neutropenia, grade 3-4 thrombocytopenia or anemia and grade 3-4 non-hematological toxicity.

During treatment, patients were observed daily by their radiation oncologist and underwent weekly physical examinations as well as assessments of weight. Blood was tested for routine analysis at least once weekly, while liver and renal function were assessed every 2 wk. Antacid and gastric mucosa protectants were administered on a prophylactic basis. Anti-emetics and antidiarrheal agents were prescribed when needed.

Statistical analysis

The trial was designed using a conventional dose-escalation schema with the primary endpoint of defining the MTD of capecitabine when combined with IMRT. The second endpoint was about the calculation of overall survival (OS), which was defined from the date of surgery to the date of death or last follow-up. Locoregional recurrence (LRR) was defined as any recurrence in the tumor bed, anastomoses, stumps, gastric remnant or a recurrence in the regional lymphatics and locoregional control (LRC) was calculated accordingly. Survival curves were calculated with the Kaplan-Meier method by means of the SPSS for Windows program, version 15.0 (SPSS, Chicago, Illinois, United States).

RESULTS

Between October 2007 and August 2009, 18 patients (12 men, 6 women) were enrolled in the study. The patient characteristics are presented in Table 2. The median age was 54 years (range, 29-66 years). All patients had metastatic LNs; the median number of positive LNs was 6 (range, 1-15), and the total number of resected LNs was 19 (range, 5-35). Twelve patients underwent R0 resection (66.7%). Fifteen patients received adjuvant chemotherapy under the leucovorin, fluorouracil and oxaliplatin (FOLFOX4) regimen, with a median number of 6 cycles (range, 3-11); of these 15 patients, seven received FOLFOX4 therapy before CRT, and the rest received it after CRT.

Dose escalation and toxicity

Six patients each were enrolled at dose levels Ⅰ, Ⅱ and

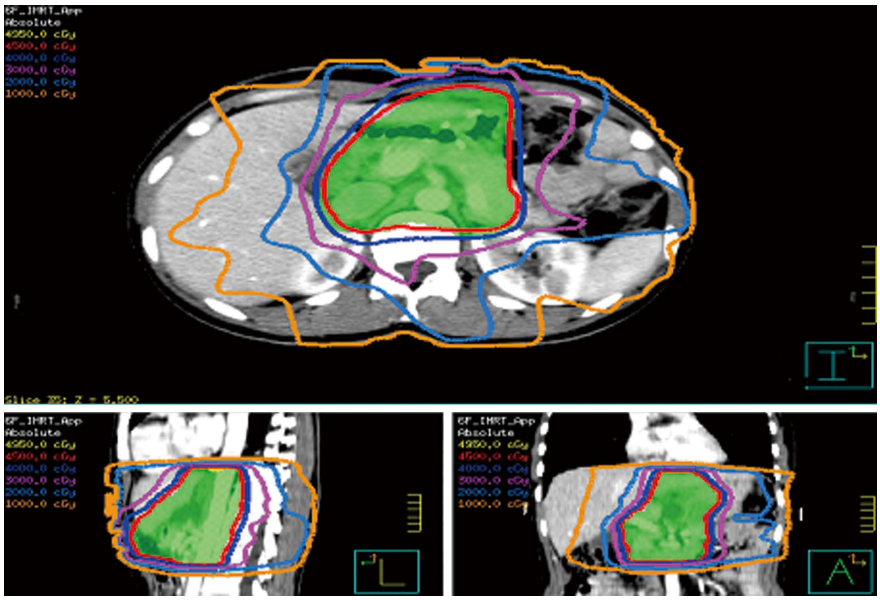


Figure 1 Distribution of 6-field intensity modulated radiotherapy plan. Isodose distributions are shown in three orthogonal planes through the middle of the planning target volume (PTV). The isodose levels are shown colored lines over the computed tomography images, with the green area indicating the PTV.

Table 2 Characteristics of the patients (*n* = 18) *n* (%)

Characteristic	Value
Median (range)	54 (29-66)
Men	12 (66.7)
Location of primary tumor	
Upper 1/3	4 (22.2)
Middle 1/3	2 (11.1)
Lower 1/3	12 (66.7)
Surgery type	
Proximal partial gastrectomy	4 (22.2)
Distal partial gastrectomy	12 (66.7)
Total gastrectomy	2 (11.1)
Extent of dissection	
R0 (D1, D2)	12 (9, 3) (66.7)
R1	2 (11.1)
R2	4 (22.2)
Tumor differentiation	
Well	1 (5.6)
Moderately	3 (16.7)
Poorly	14 (77.8)
Signet ring cell	
Yes	9 (50.0)
No	9 (50.0)
Adjuvant chemotherapy	
Yes	15 (83.3)
No	3 (16.7)
Stage (AJCC 7th)	
Ⅱ	3 (16.7)
Ⅲ	15 (83.3)

AJCC: American Joint Committee on Cancer.

Table 3 Dose-limiting toxicities of chemoradiotherapy

Level	Capecitabine (mg/m^2, bid)	*n*	No. patients with the DLT	DLT (G3/4)	RT dose when DLT occurred (Gy)
Ⅰ	625	6	1	Nausea, vomiting, anorexia	5.4
Ⅱ	700	6	1	Nausea, anorexia	45
Ⅲ	800	6	1	Neutropenia	36
			1	Radiation esophagitis	43.2

DLT: Dose-limiting toxicity; RT: Radiotherapy.

Ⅲ. Grade 3 anorexia and nausea and grade 4 vomiting were observed in one of six patients at the first level after only three fractions of radiation had been performed. At level Ⅱ, one of the first three patients encountered grade 3 anorexia and nausea. After upgrading to level Ⅲ, one patient developed grade 4 neutropenia, and another patient of the subsequent three patients developed grade 3 radiation esophagitis. The trial was then ended, and no patient was upgraded to levels Ⅳ and Ⅴ. Therefore, level Ⅲ (capecitabine, 800 mg/m^2, twice daily) was determined to be the MTD. The DLTs met at levels Ⅰ, Ⅱ and Ⅲ (Table 3) were grade 3 anorexia and nausea (levels Ⅰ and Ⅱ), grade 4 vomiting (level Ⅰ), grade 4 neutropenia and grade 3 radiation esophagitis (level Ⅲ).

Grade 1-3 leukopenia (16 patients, 88.9%), anorexia (15, 83.3%) and nausea (15, 83.3%) were the most common toxicities. No renal or liver toxicity occurred in any patient. The detected grade 1-4 toxicities have been shown in Table 4.

Of the 12 patients with R0 resection, 45 Gy was delivered to nine patients as planned, including one patient who developed a DLT at level Ⅱ but finally completed the entire treatment protocol. The remaining three patients completed CRT with 5.4, 36 and 43.2 Gy, owing to the occurrence of DLTs. Of the six patients with R1/R2 resection, two received sequential boosts for a total dose of 55.8 Gy without any DLTs. The remaining patients did not receive boosts because of difficulty in contouring the location of the residual tumor area without the placement of clips during surgery.

Survival and relapse

During a median follow-up of 45 mo (range, 5-58 mo), five patients died: four of progression of gastric cancer

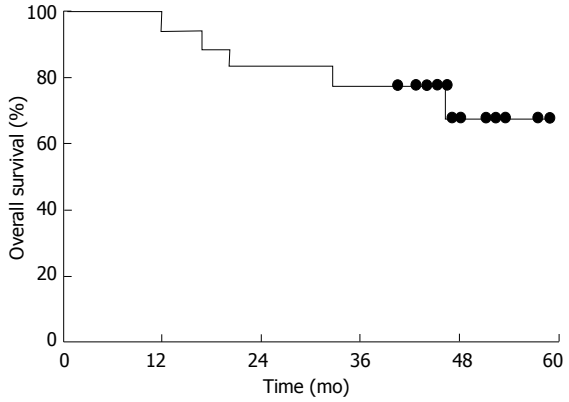


Figure 2 Overall survival rates, according to the Kaplan-Meier technique.

and one of pulmonary embolism. The 4-year LRC and OS rates were 93.8% and 68.1%, respectively (Figure 2).

Table 4 Grade 1-4 toxicities *n* (%)

Toxicity	Grade 1-2	Grade 3-4
Nausea	13 (72.2)	2 (11.1)
Vomiting	5 (27.8)	1 (5.6)
Diarrhea	2 (11.1)	0
Stomatitis	3 (16.7)	0
Anorexia	13 (72.2)	2 (11.1)
Fatigue	13 (72.2)	0
Weight loss	7 (38.9)	0
HFS	3 (16.7)	0
Esophagitis	0	1 (5.6)
Leukopenia	14 (77.8)	2 (11.1)
Neutropenia	3 (16.7)	1 (5.6)
Anemia	3 (16.7)	0
Thrombocytopenia	5 (27.8)	0
ALT/AST	0	0
Renal	0	0

HFS: Hand foot syndrome; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

DISCUSSION

Our results suggest that postoperative CRT with oral capecitabine and 45 Gy IMRT was well tolerated in locally advanced, gastric cancer patients after a partial or total gastrectomy. The most frequent adverse events were leukopenia, anorexia and nausea, although most of these were at grade 1 or 2. DLTs included grade 3 nausea, anorexia and radiation esophagitis and grade 4 neutropenia and vomiting. The MTD of oral capecitabine was determined to be 800 mg/m^2 twice daily.

The benefit of postoperative CRT for locally advanced gastric cancer remains controversial. The benefits or drawbacks of this treatment mainly depend on whether a D2 or D0-1 lymphadenectomy has been performed. Differing results regarding D1 or D2 lymphadenectomy have been reported from both Western and Eastern studies[19-21]. Two large, randomized, clinical trials, INT 0116 and the ARTIST study, have provided compelling evidence on this topic. Since INT 0116 was published in 2001, the application of concurrent CRT has become widespread. This was the first study to provide evidence demonstrating that combined CRT following R0 resection and D0/D1 lymphadenectomy improves disease-free survival (DFS) and OS. Even after 10 years of follow-up, updated aanalysis of the INT 0116 study still show a strong, persistent benefit of adjuvant CRT in terms of DFS and OS, because the reduction of LRR may reduce the overall relapse in the majority of patients[22]. Recently, a large, prospective, randomized trial (ARTIST) from Korea indicated a 3-year DFS benefit for postoperative, concurrent CRT in patients with positive LNs after R0 resection and D2 lymphadenectomy compared with those who received chemotherapy alone[7]. Although this result was obtained via a subgroup analysis, further studies that focus on LN-positive patients with R0 resection and D2 lymphadenectomy are warranted. Owing to its remarkable control of LRR, fluoropyrimidine-based postoperative CRT following R1 or R2 resection is unquestionably a recommended and effective treatment for gastric cancer according to NCCN guidelines[6].

In our specialized cancer hospital, most surgeons perform D1 or D2 lymphadenectomy, which accounts for the high 5-year LRR rate of 27.6%[23]. This result is not comparable to those of studies from Japan and Korea due to the inferior performance of the D2 lymphadenectomy[7,11,24]; therefore, postoperative CRT should be introduced in our hospital as a standard of care. Unfortunately, this regimen is still not well accepted or routinely performed. One of the reasons for this might be the concern about the high rate of side effects of postoperative CRT based on the INT 0116 report.

In the INT 0116 study, 54% and 32% patients developed grade 3/4 hematological and gastrointestinal toxicities, respectively. Three patients (1%) suffered toxic deaths, and 31% did not complete the treatment due to toxicities[4]. However, these outcomes were obtained in the era of two-dimensional RT with a large AP-PA field. We currently utilize two methods to avoid these outcomes. Firstly, 3DCRT or IMRT provides excellent coverage of the target volume while avoiding normal tissue. Leong *et al*[14] reported that 3DCRT provides more adequate coverage of the target volume with 99% PTV receiving 95% of the prescribed dose, compared to 93% PTV using AP-PA fields. The doses to the kidneys and spinal cord were much lower with the conformal technique. Furthermore, IMRT could deliver more efficient doses to the target volume while reducing the dose to the kidneys when compared with the conventional technique[25].

Secondly, the exclusion of the remnant stomach from the radiation field could significantly reduce the acute side effects without compromising long-term survival rates (DFS, with remnant stomach irradiated *vs* without, 70.4% *vs* 71.0%, P = NS; OS, 72.3% *vs* 72.9%, P = NS)[26]. Our analysis also revealed that only 4.7% out of 297 patients with locally advanced gastric cancer developed a recurrence in the remnant stomach after a D1 or D2 lymphadenectomy[23].

More importantly, capecitabine was demonstrated to be a safer and more effective regimen than 5FU, which was used as part of the concurrent chemotherapy regimen in INT 0116. In the past 10 years, capecitabine has been implemented extensively in the treatment of colorectal, breast and gastric cancer, either as a single agent or combined with other chemotherapeutic and targeted agents. Oral capecitabine has shown comparable results to those of 5FU infusion, with a much safer side effects profile and without the need for an invasive delivery route. Therefore, capecitabine has been recognized as a standard of care for the treatment of advanced gastric cancer worldwide[6,27,28]. Moreover, a German, randomized, non-inferiority, phase Ⅲ trial of 392 rectal cancer patients concluded that capecitabine could replace 5FU in adjuvant or neoadjuvant CRT regimens for patients with locally advanced rectal cancer, with a non-inferior OS (P = 0.0004), a significantly lower distant metastasis rate (P = 0.04) and better DFS rate (P = 0.07)[28].

The combined current literature indicates that postoperative CRT is a good option for patients with locally advanced gastric cancer who have undergone R0 and D0-1 lymphadenectomy. To avoid acute side effects and make the drug safer, a combination of an appropriate irradiation field, and the use of advanced techniques such as 3DCRT or IMRT and mature chemotherapy regimens with capecitabine is highly recommended.

In the present study, we considered that postoperative CRT with 800 mg/m^2 oral capecitabine twice daily combined with IMRT with a dose of at least 45 Gy in 25 fractions was feasible and safe. The recommended dose of capecitabine was similar to that used in the RT phase of the CRT group in the ARTIST trial (capecitabine, 825 mg/m^2 twice daily during RT treatment)[7]. Although the number of patients in our study was limited, the results of a 4-year follow-up show LRC and OS rates as high as 93.8% and 68.1%, respectively, which are very promising. A phase Ⅱ study is ongoing, and its results are eagerly awaited.

COMMENTS

Research frontiers

Gastric cancer has the third highest mortality rate of all cancers in China. The optimal treatment is a R0 resection, but this can be offered to < 50% patients because of advanced disease. Chemoradiotherapy can be offered to these patients, but side effects can be severe.

Research frontiers

The 5-fluorouracil (5FU) analog, capecitabine, has been widely used for gastric cancer treatment and is comparable to the widely used drug, 5FU. The use of intensity modulated radiotherapy (IMRT) is also known to be superior to standard radiotherapy, but the combined use of both capecitabine and IMRT for gastric cancer has not been investigated.

Innovations and breakthroughs

Postoperative capecitabine (800 mg/m^2) with IMRT of at least 45 Gy for 25 fractions was found to be safe and feasible. Although the number of patients in their study (n = 18) was limited, the results of a 4-year follow-up show locoregional control and overall survival rates as high as 93.8% and 68.1%, respectively, which are very promising.

Applications

Further work is required to establish the range of side effects with the use of capecitabine in combination with IMRT. The dose limiting toxicities observed in this study were anorexia, nausea and vomiting, neutropenia and radiation esophagitis. A Phase Ⅱ study will then be established to determine the treatment efficacy of this regimen.

Terminology

IMRT is a method of radiotherapy that is delivering radiation to precise tissue areas that is greatly superior to conventional radiotherapy as it spares more normal tissue outside the radiation field.

Peer review

This is a very interesting phase Ⅰ trial about capecitabine combined with IMRT for locally advanced gastric cancer. Although the small number of patients involved, the trial was well done and the conclusions are according to another trial published and are correct. This is an interesting issue because utilize IMRT in the treatment for locally advanced gastric cancer and it will be very important to do a phase Ⅱ trial.

REFERENCES

1 **Jemal A**, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011; **61**: 69-90 [PMID: 21296855 DOI: 10.3322/caac.20107]

2 **He J**, Chen WQ. Chinese Cancer Registry Annual Report, 2012. Beijing: Military Medical Science Press, 2012: 28-30

3 **Zhan YQ**, Li W, Sun XW, Chen YB, Xu L, Chen G, Guan YX, Li YF, Xu DZ, Sun XF, Zhang HZ, Lin ZW. [Long-term results of surgical treatment of stomach cancer: clinical experience of forty years from Sun Yat-sen University Cancer Center]. *Zhonghua Waike Zazhi* 2005; **43**: 1109-1113 [PMID: 16194306]

4 **Macdonald JS**, Smalley SR, Benedetti J, Hundahl SA, Estes NC, Stemmermann GN, Haller DG, Ajani JA, Gunderson LL, Jessup JM, Martenson JA. Chemoradiotherapy after surgery compared with surgery alone for adenocarcinoma of the stomach or gastroesophageal junction. *N Engl J Med* 2001; **345**: 725-730 [PMID: 11547741 DOI: 10.1056/NEJMoa010187]

5 **Solomon NL**, Cheung MC, Byrne MM, Zhuge Y, Franceschi D, Livingstone AS, Koniaris LG. Does chemoradiotherapy improve outcomes for surgically resected adenocarcinoma of the stomach or esophagus? *Ann Surg Oncol* 2010; **17**: 98-108 [PMID: 19777191 DOI: 10.1245/s10434-009-0679-y]

6 **NCCN Guidelines Panel**. National Comprehensive Cancer Network Guidelines in Oncology (NCCN Guidelines). Gastric cancer. 2013. Version 2, 2013-04-25; GAST-3: 11 screens. Available from: URL: http://www.nccn.org/index.asp

7 **Lee J**, Lim do H, Kim S, Park SH, Park JO, Park YS, Lim HY, Choi MG, Sohn TS, Noh JH, Bae JM, Ahn YC, Sohn I, Jung SH, Park CK, Kim KM, Kang WK. Phase III trial comparing capecitabine plus cisplatin versus capecitabine plus cisplatin with concurrent capecitabine radiotherapy in completely resected gastric cancer with D2 lymph node dissection: the ARTIST trial. *J Clin Oncol* 2012; **30**: 268-273 [PMID: 22184384 DOI: 10.1200/JCO.2011.39.1953]

8 **Kang YK**, Kang WK, Shin DB, Chen J, Xiong J, Wang J, Lichinitser M, Guan Z, Khasanov R, Zheng L, Philco-Salas M, Suarez T, Santamaria J, Forster G, McCloud PI. Capecitabine/cisplatin versus 5-fluorouracil/cisplatin as first-line therapy in patients with advanced gastric cancer: a randomised phase III noninferiority trial. *Ann Oncol* 2009; **20**: 666-673 [PMID: 19153121 DOI: 10.1093/annonc/mdn717]

9 **Jansen EP**, Boot H, Dubbelman R, Bartelink H, Cats A, Verheij M. Postoperative chemoradiotherapy in gastric cancer -- a Phase I/II dose-finding study of radiotherapy with dose escalation of cisplatin and capecitabine chemotherapy. *Br J Cancer* 2007; **97**: 712-716 [PMID: 17848909 DOI: 10.1038/sj.bjc.6603965]

10 **Hong YS**, Song SY, Lee SI, Chung HC, Choi SH, Noh SH, Park JN, Han JY, Kang JH, Lee KS, Cho JY. A phase II trial of capecitabine in previously untreated patients with advanced and/or metastatic gastric cancer. *Ann Oncol* 2004; **15**:

1344-1347 [PMID: 15319239 DOI: 10.1093/annonc/mdh343]
11 **Bang YJ**, Kim YW, Yang HK, Chung HC, Park YK, Lee KH, Lee KW, Kim YH, Noh SI, Cho JY, Mok YJ, Kim YH, Ji J, Yeh TS, Button P, Sirzén F, Noh SH. Adjuvant capecitabine and oxaliplatin for gastric cancer after D2 gastrectomy (CLASSIC): a phase 3 open-label, randomised controlled trial. *Lancet* 2012; **379**: 315-321 [PMID: 22226517 DOI: 10.1016/S0140-6736(11)61873-4]
12 **Ringash J**, Perkins G, Brierley J, Lockwood G, Islam M, Catton P, Cummings B, Kim J, Wong R, Dawson L. IMRT for adjuvant radiation in gastric cancer: a preferred plan? *Int J Radiat Oncol Biol Phys* 2005; **63**: 732-738 [PMID: 15978742 DOI: 10.1016/j.ijrobp.2005.03.013]
13 **Lohr F**, Dobler B, Mai S, Hermann B, Tiefenbacher U, Wieland P, Steil V, Wenz F. Optimization of dose distributions for adjuvant locoregional radiotherapy of gastric cancer by IMRT. *Strahlenther Onkol* 2003; **179**: 557-563 [PMID: 14509955 DOI: 10.1007/s00066-003-1087-z]
14 **Leong T**, Willis D, Joon DL, Condron S, Hui A, Ngan SY. 3D conformal radiotherapy for gastric cancer--results of a comparative planning study. *Radiother Oncol* 2005; **74**: 301-306 [PMID: 15763311 DOI: 10.1016/j.radonc.2005.01.006]
15 **Kassam Z**, Lockwood G, O'brien C, Brierley J, Swallow C, Oza A, Siu L, Knox JJ, Wong R, Cummings B, Kim J, Moore M, Ringash J. Conformal radiotherapy in the adjuvant treatment of gastric cancer: Review of 82 cases. *Int J Radiat Oncol Biol Phys* 2006; **65**: 713-719 [PMID: 16626887 DOI: 10.1016/j.ijrobp.2006.01.001]
16 **Jansen EP**, Boot H, Saunders MP, Crosby TD, Dubbelman R, Bartelink H, Verheij M, Cats A. A phase I-II study of postoperative capecitabine-based chemoradiotherapy in gastric cancer. *Int J Radiat Oncol Biol Phys* 2007; **69**: 1424-1428 [PMID: 17689023 DOI: 10.1016/j.ijrobp.2007.05.004]
17 **Edge SB**, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A, editors. AJCC cancer staging manual (7th edition). New York: Springer, 2010: 145-152
18 **Japanese Gastric Cancer Association.** Japanese Classification of Gastric Carcinoma - 2nd English Edition - *Gastric Cancer* 1998; **1**: 10-24 [PMID: 11957040]
19 **Wu CW**, Hsiung CA, Lo SS, Hsieh MC, Chen JH, Li AF, Lui WY, Whang-Peng J. Nodal dissection for patients with gastric cancer: a randomised controlled trial. *Lancet Oncol* 2006; **7**: 309-315 [PMID: 16574546 DOI: 10.1016/S1470-2045(06)70623-4]
20 **Sasako M**, Sano T, Yamamoto S, Kurokawa Y, Nashimoto A, Kurita A, Hiratsuka M, Tsujinaka T, Kinoshita T, Arai K, Yamamura Y, Okajima K. D2 lymphadenectomy alone or with para-aortic nodal dissection for gastric cancer. *N Engl J Med* 2008; **359**: 453-462 [PMID: 18669424 DOI: 10.1056/NEJMoa0707035]
21 **Bonenkamp JJ**, Hermans J, Sasako M, van de Velde CJ, Welvaart K, Songun I, Meyer S, Plukker JT, Van Elk P, Obertop H, Gouma DJ, van Lanschot JJ, Taat CW, de Graaf PW, von Meyenfeldt MF, Tilanus H. Extended lymph-node dissection for gastric cancer. *N Engl J Med* 1999; **340**: 908-914 [PMID: 10089184 DOI: 10.1056/NEJM199903253401202]
22 **Smalley SR**, Benedetti JK, Haller DG, Hundahl SA, Estes NC, Ajani JA, Gunderson LL, Goldman B, Martenson JA, Jessup JM, Stemmermann GN, Blanke CD, Macdonald JS. Updated analysis of SWOG-directed intergroup study 0116: a phase III trial of adjuvant radiochemotherapy versus observation after curative gastric cancer resection. *J Clin Oncol* 2012; **30**: 2327-2333 [PMID: 22585691 DOI: 10.1200/JCO.2011.36.7136]
23 **Wang X**, Jin J, Li YX, Wang SL, Wang WH, Song YW, Liu YP, Liu XF, Yu ZH. Analysis of recurrence for locally advanced gastric or gastroesophageal cancer patients after receiving curative gastrectomy (>D1) and its indication for adjuvant chemoradiotherapy. *Zhonghua Fangshe Zhongliuxue Zazhi* 2011; **20**: 133-141
24 **Sakuramoto S**, Sasako M, Yamaguchi T, Kinoshita T, Fujii M, Nashimoto A, Furukawa H, Nakajima T, Ohashi Y, Imamura H, Higashino M, Yamamura Y, Kurita A, Arai K. Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. *N Engl J Med* 2007; **357**: 1810-1820 [PMID: 17978289 DOI: 10.1056/NEJMoa072252]
25 **Wieland P**, Dobler B, Mai S, Hermann B, Tiefenbacher U, Steil V, Wenz F, Lohr F. IMRT for postoperative treatment of gastric cancer: covering large target volumes in the upper abdomen: a comparison of a step-and-shoot and an arc therapy approach. *Int J Radiat Oncol Biol Phys* 2004; **59**: 1236-1244 [PMID: 15234061 DOI: 10.1016/j.ijrobp.2004.02.051]
26 **Nam H**, Lim do H, Kim S, Kang WK, Sohn TS, Noh JH, Kim YI, Park CH, Park CK, Ahn YC, Huh SJ. A new suggestion for the radiation target volume after a subtotal gastrectomy in patients with stomach cancer. *Int J Radiat Oncol Biol Phys* 2008; **71**: 448-455 [PMID: 18234442 DOI: 10.1016/j.ijrobp.2007.09.055]
27 **NCCN Guidelines Panel.** National Comprehensive Cancer Network Guidelines in Oncology (Chinese version). Gastric cancer. 2011. Version 1, GAST-2: 9 screens. Available from: URL: http: //www.nccnchina.org/nccn-guidelines-china.aspx
28 **Hofheinz RD**, Wenz F, Post S, Matzdorff A, Laechelt S, Hartmann JT, Müller L, Link H, Moehler M, Kettner E, Fritz E, Hieber U, Lindemann HW, Grunewald M, Kremers S, Constantin C, Hipp M, Hartung G, Gencer D, Kienle P, Burkholder I, Hochhaus A. Chemoradiotherapy with capecitabine versus fluorouracil for locally advanced rectal cancer: a randomised, multicentre, non-inferiority, phase 3 trial. *Lancet Oncol* 2012; **13**: 579-588 [PMID: 22503032 DOI: 10.1016/S1470-2045(12)70116-X]

P- Reviewers: Araujo A, Cihan YB, Tunio MA
S- Editor: Gou SX **L- Editor**: A **E- Editor**: Wu HL

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.1074

World J Gastroenterol 2014 January 28; 20(4): 1074-1078
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Expression of granulocyte colony-stimulating factor receptor in rectal cancer

Xiao-Dong Yang, Ping Huang, Feng Wang, Ze-Kuan Xu

Xiao-Dong Yang, Ping Huang, Feng Wang, Ze-Kuan Xu, Department of General Surgery, the First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, Jiangsu Province, China
Author contributions: Yang XD and Huang P designed the research; Yang XD, Huang P and Xu ZK performed experiments and interpreted the data; Yang XD and Wang F wrote the paper.
Correspondence to: Ping Huang, MD, Department of General Surgery, the First Affiliated Hospital of Nanjing Medical University, No. 300, Guangzhou Road, Nanjing 210029, Jiangsu Province, China. huangpingdoctor@163.com
Telephone: +86-25-83718836 Fax: +86-25-83718836
Received: August 5, 2013 Revised: October 25, 2013
Accepted: November 12, 2013
Published online: January 28, 2014

Abstract

AIM: To evaluate whether granulocyte colony-stimulating factor receptor (G-CSFR) expression before preoperative irradiation can predict the radiosensitivity of rectal cancer.

METHODS: The expression of G-CSFR was examined, using immunohistochemistry, in biopsy specimens from 126 patients with locally advanced rectal adenocarcinoma before preoperative irradiation. Radiosensitivity was then evaluated according to the Rectal Cancer Regression Grading. Endoscopic inspection was used to detect the tumor area in each patient. General patient information, such as age, gender, lymph node status, tumor size and degree of differentiation was recorded. A statistical analysis was then performed to evaluate the correlation between clinical or pathological parameters and G-CSFR expression in tumors.

RESULTS: According to endoscopic inspection, the tumor area ranged from 4 to 48 cm^2 (median, 15 cm^2). Positive G-CSFR immunoreactions (G-CSFR$^+$) were observed in 85 specimens, and negative (G-CSFR$^-$) in 41. No significant differences were found in age, gender, tumor invasion, lymph node status and tumor size between G-CSFR$^+$ and G-CSFR$^-$ patients. G-CSFR expression was positively correlated with poor radiotherapy response (58.8% *vs* 75.6%, $P = 0.014$, $r = 0.219$). The proportion of well-differentiated tumors in G-CSFR$^+$ and G-CSFR$^-$ patients was 24.7% and 36.6%, respectively. Sphincter preservation was observed in 57.6% of G-CSFR$^+$ patients and 78.5% of G-CSFR$^-$ patients. Significant correlations were found between G-CSFR expression and tumor differentiation (24.7% *vs* 36.6%, $P = 0.019$, $r = 0.210$), as well as sphincter preservation (57.6% *vs* 78.5%, $P = 0.044$, $r = 0.180$).

CONCLUSION: The expression of G-CSFR before preoperative irradiation may predict the radiosensitivity of rectal cancer.

Key words: Radiotherapy; Rectal cancer; Radiosensitivity; Predictive factor; Tumor

Core tip: It is unknown whether the expression of granulocyte colony-stimulating factor receptor (G-CSFR) can predict tumor response or sphincter preservation in patients with rectal cancer receiving preoperative radiotherapy. This study found that the expression of G-CSFR before preoperative irradiation may predict the radiosensitivity of rectal cancer.

Yang XD, Huang P, Wang F, Xu ZK. Expression of granulocyte colony-stimulating factor receptor in rectal cancer. *World J Gastroenterol* 2014; 20(4): 1074-1078 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/1074.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.1074

INTRODUCTION

Colorectal cancer is one of the most common can-

cers in humans and the second leading cause of cancer death[1,2]. Each year there are approximately 42000 newly diagnosed cases in the United States alone, and 1 million worldwide[3]. As the main treatment option is confined to surgery, preoperative radiotherapy has been used to improve the outcome of surgery in patients with rectal cancer. Studies have shown that the local recurrence rate was 20%-60% in patients with locally advanced rectal cancer treated with surgery alone, whereas adjuvant radiotherapy improved local control and survival rate[3]. Other studies also showed the benefits of preoperative radiotherapy[4,5]. When comparing the benefits of neo-adjuvant radiotherapy with adjuvant radiotherapy, the former therapy is superior because it decreases tumor bulk, enhances sphincter preservation and reduces acute toxicity. As a result, preoperative pelvic radiotherapy resulted in a 4%-30% pathological complete response and a partial response in 30%-60% of cases[3-5].

Partial response after neo-adjuvant radiation therapy occurs in 46.7% of patients, and of these patients, 17.9% have complete responses. A previous study demonstrated that response to preoperative radiotherapy varies, depending on clinical factors such as tumor stage, total dose, fractionation schedules, concomitant chemotherapy treatment or time between radiation and surgery[6]. The study also suggested that although tumor-node-metastasis classification was useful for staging patients and selecting them for specific treatments, it was not sufficient: patients at the same stage may have different outcomes[6]. Therefore, additional prognostic biomarkers are needed for the clinical management of patients with rectal cancer. Significant research effort has been devoted to developing molecular targeted therapies or searching for molecular markers that are useful either in predicting treatment outcome or in selecting patients for specific molecular targeted therapies, based on particular tumor characteristics[7].

A previous study in our laboratory demonstrated that granulocyte colony-stimulating factor receptor (G-CSFR) was expressed in most human colorectal cancers, was involved in the development of human colorectal cancer and may be associated with more aggressive cancer[8]. However, little data on the effect of G-CSFR expression on response to pelvic radiotherapy in the preoperative setting are available. Thus, in the present study, we determined the expression level of G-CSFR in locally advanced rectal cancer and assessed whether G-CSFR expression could predict tumor response in patients treated with preoperative radiation therapy.

MATERIALS AND METHODS

Patient selection and pretreatment evaluation

All patients gave informed consent before their inclusion in the study, and the Ethics Committee of the hospital approved all the human studied, which were performed in accordance with the ethical standards. The inclusion criteria were as follows: (1) histologically confirmed diagnosis of adenocarcinoma of the rectum, with the inferior margin of the tumor less than 10 cm from the anal verge; (2) clinically staged as locally advanced rectal cancer; (3) administration of preoperative radiotherapy followed by surgical resection; and (4) available tissue samples of the diagnostic biopsy and tumor specimen for review and immunostaining. From September 2002 to October 2007, 126 patients with locally advanced rectal adenocarcinoma were administered preoperative radiotherapy in the First Affiliated Hospital of Nanjing Medical University (Nanjing, China). All patients were free of distant metastases at the time of diagnosis. Assessment of local extension was based on clinical and radiographic evaluations. Diagnostic studies consisted of colorectal endoscopy, abdominopelvic computed tomography (CT), chest X-ray, endoscopic ultrasonography and routine laboratory studies. The patients were staged according to the American Joint Committee on Cancer Staging.

Immunohistochemical assay of G-CSFR

Consecutive sections (4 μm) of formalin-fixed and paraffin-embedded tissue specimens were used for immunohistochemistry. The sections were stained using a labeled streptavidin-biotin peroxidase method (LSAB2 Kit; Dako Japan Inc., Kyoto, Japan) comprising a mouse monoclonal anti-G-CSFR antibody (Serotec Ltd, United Kingdom; dilution 1:80). The slides were immersed in 0.3% hydrogen peroxide/methanol for 10 min to deplete endogenous peroxidase. Then, nonspecific binding sites were blocked with 0.3% normal goat serum for 10 min. The primary antibody was then applied, and the sections were incubated overnight at 4 ℃. After washing with phosphate-buffered saline (PBS, 0.01 mol/L pH 7.4), the secondary antibody (biotinylated goat-anti-mouse IgG) was applied to the tissue sections, which were incubated at room temperature for 10 min. After washing with PBS, a streptavidin peroxidase reagent was applied and the sections were incubated at room temperature for 10 min. Finally, the reaction product was visualized by developing the color following incubation of the slides in a solution of 0.3% hydrogen peroxide and 3-amino-9-ethylcarbazole chromogen. The sections were lightly counterstained with hematoxylin. Negative controls were parallel sections in which the primary antibody was replaced with the same volume of PBS. The immunoreactions were graded as negative (0), weakly positive (1), moderately positive (2), or strongly positive (3), according to the immunostaining intensity. Two independent pathologists who had no preknowledge of this study read all the slides. In the case of disagreement between the two pathologists, a third pathologist read the slides, discussed the results with his/her two colleagues, and then made the final decision.

Preoperative radiotherapy and surgical modalities

Physical examination, endorectal ultrasound and pelvic CT were used to stage the patients. Preoperative radiotherapy was delivered to the pelvis with 60Co. The clinical target volume included the tumor, the entire rectum,

Table 1 Relationship between granulocyte colony-stimulating factor receptor expression and clinicopathological factors

Clinicopathological parameters	Cases	G-CSFR				*P* value	*r*
		0	1	2	3		
		41	26	28	31		
Age (yr)							
< 60	67	20	17	15	15		
⩾ 60	59	21	9	13	16	NS	
Gender							
Male	54	18	10	11	15		
Female	72	23	16	17	16	NS	
Pathological stage							
LN^-	72	21	12	20	19		
LN^+	54	20	14	8	12	NS	
T3	38	13	6	9	10		
T4	88	28	20	19	21	NS	
Size (cm^2)							
> 15	66	18	14	15	19		
⩽ 15	60	23	12	13	12	NS	
Differentiation							
Well	36	15	6	10	5		
Moderate	55	17	15	13	10		
Poor	35	9	5	5	16	0.019	0.210
Sphincter preservation							
Yes	81	32	15	17	17		
No	45	9	11	11	14	0.044	0.180
Distance from anal verge (cm)							
5-10	82	28	17	19	18		
< 5	44	13	9	9	13	NS	
Pathological response							
Complete	37	18	7	5	7		
Partial	44	13	10	11	10		
No response	45	10	9	12	14	0.014	0.219

G-CSFR: Granulocyte colony-stimulating factor receptor; NS: Not significant; LN: Lymph nodes.

the anterior wall of the sacrum, the posterior wall of the prostate or vagina, and the perirectal, presacral, hypogastric, actuator, and iliac lymph nodes. The standard anterior-posterior/posterior-anterior fields received 45 Gy, and then two opposed lateral boost fields up to 50.4 Gy were used. All patients received conventional fractionation of 1.80 Gy/d, and five fractions per week. Physical examination, endorectal ultrasound and pelvic CT were used to restage the patients four to six weeks later. The patients then received total mesorectal excision by a group of surgeons in our hospital. Subsequently, the pathological response was evaluated in postsurgical specimens and graded according to the method described by Mandard *et al*[9]. This method was used to assess the pathological response after neo-adjuvant radiotherapy in rectal cancer on a scale of 1-5, based on the presence of residual tumor cells and the extent of fibrosis. The two pathologists who were blinded to the study assessed the grade of tumor response. We considered grade 1, which is defined as the absence of residual tumor cells and fibrosis extending through the layers of the rectum, as "complete pathological response". We considered grade 2-4, which are characterized by the presence of various amounts of residual tumor cells, as "partial response", and grade 5, defined as absence of tumor response, as "no response".

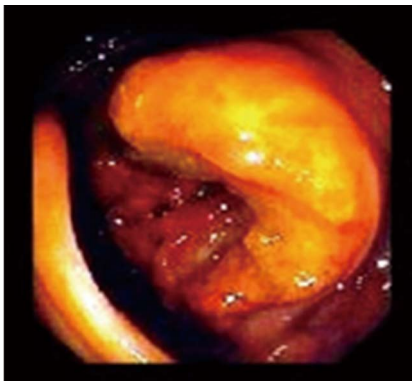


Figure 1 Pre-radiotherapeutic tumor under endoscopy.

Statistical analysis

The Kendall's tau-b and the Spearman test were used to examine the correlation between various clinical or pathological parameters and the expression of G-CSFR in tumors. The correlation was evaluated using the Spearman test. *P* values less than 0.05 were considered statistically significant. All calculations were performed using SPSS 12.0 for Windows.

RESULTS

General patient information, such as age, gender, lymph node status, tumor size and degree of differentiation are summarized in Table 1. There were 54 men and 72 women, aged from 30 to 90 years (median, 60 years). According to the endoscopic inspection, the tumor area ranged from 4 to 48 cm^2 (median, 15 cm^2) (Figure 1). The results of G-CSFR immunostaining are also summarized in Table 1. Positive immunoreactions ($G\text{-}CSFR^+$) of cytoplasmic staining in tumor cells were observed in 85 of 126 pre-radiation biopsy specimens, while 41 were negative ($G\text{-}CSFR^-$). No significant differences were found in age, gender, tumor invasion, lymph node status and tumor size between $G\text{-}CSFR^+$ patients and $G\text{-}CSFR^-$ patients. There were 36 well differentiated tumors, 55 moderately differentiated tumors and 35 poorly differentiated tumors.

Significant correlations were found for the pathological response, tumor differentiation and the proportion of sphincter preservation between $G\text{-}CSFR^+$ patients and $G\text{-}CSFR^-$ patients. In this study, the proportion of sphincter preservation was 57.6% (49/85) in $G\text{-}CSFR^+$ patients, compared to 78.5% (32/41) in $G\text{-}CSFR^-$ patients ($P = 0.044$, $r = 0.180$). The proportion of well differentiated tumors in $G\text{-}CSFR^+$ patients was 24.7% (21/85), while that in $G\text{-}CSFR^-$ patients was 36.6% (15/41) ($P = 0.019$, $r = 0.210$). The distance from the anal verge to the inferior margin of the tumor was 5 - 10 cm in 82 patients, whereas the distance was less than 5 cm in 44 patients. Various responses to radiotherapy were

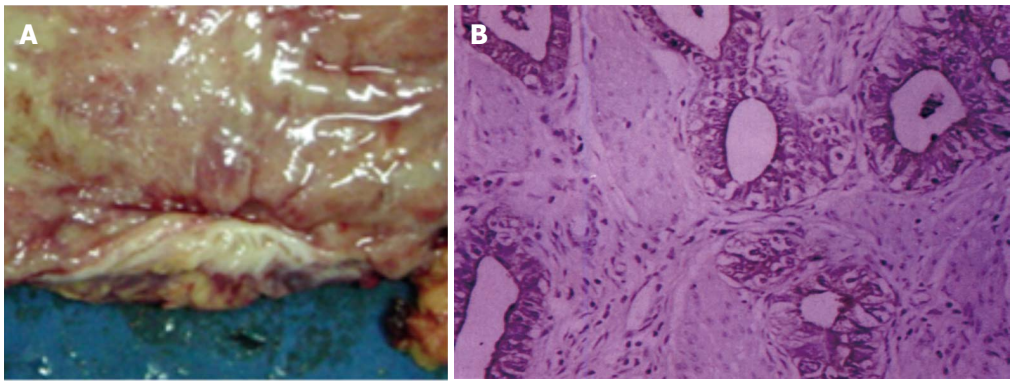


Figure 2 Post-radiotherapeutic tumor. A: The original tumor shrank and the level of peripheral necrosis was elevated; B: Complete response to radiation therapy in postoperative samples. The tumor cells were replaced by fibrosis and necrosis.

seen in the 126 post-radiation rectal cancer specimens. In hematoxylin-stained slides, 81 patients (64.3%, 81/126) responded to pelvic preoperative radiotherapy. Of these patients, 37 (29.4%, 37/126) achieved a complete pathological response, with no residual tumor in the resected specimens, and the cancer cells were completely replaced by fibrosis, necrosis, or calcified tissue (Figure 2). Partial responses were seen in 44 patients (34.9%, 44/126). Response to pelvic radiotherapy was observed in 75.6% (31/41) of G-CSFR$^-$ patients and 58.8% (50/85) of G-CSFR$^+$ patients ($P = 0.014$, $r = 0.219$). Only 19 (22.4%, 19/85) G-CSFR$^+$ patients achieved complete pathological response, compared with 43.9% (18/41) of G-CSFR$^-$ patients. Multivariate analysis showed that G-CSFR status was a significant predictor of complete remission.

DISCUSSION

Mitotic or clonogenic cell death is considered to be the major mechanism of most solid tumors response to clinical radiotherapy[10]. With increased knowledge of cell cycle regulation, apoptosis and DNA repair, attempts have been made to identify molecular markers capable of predicting the radiation sensitivity of tumors. Markers, including p53, have been studied in cell cycle regulation, apoptosis and resistance to radiation[11,12]. However, it is questionable whether these constituents can be used to predict radiotherapy response. In the present study, we followed patients in an attempt to identify a new specific molecular marker for radiotherapy sensitivity.

Our study is the first to examine the relationship between G-CSFR expression and tumor radiosensitivity in rectal cancer. The results demonstrated that G-CSFR overexpression was significantly associated with a poor response to radiotherapy, although the exact mechanism involved in this relationship remains unknown. Our previous study proved that G-CSFR expression was up-regulated in colorectal cancer cells compared with normal mucous membranes[8]. The current findings further reinforce our understanding of the mechanistic role of G-CSF/G-CSFR signaling in colorectal cancer. It is possible that the behavior of colorectal tumors expressing G-CSFR may be influenced by locally expressed G-CSF. G-CSF can be produced either by white blood cells, which frequently infiltrate colorectal tumors, or by cancer cells themselves, as shown in cultured bladder carcinoma and astrocytoma cell lines[13,14]. Downstream G-CSF signaling in non-hematopoietic cells is not well understood, although it has been intensively studied in hematopoietic cells[15]. The proliferative effect of G-CSF in colorectal cancer *in vivo* and *in vitro*, and the mechanism involved were not studied. Interestingly in our study, a significant correlation was found between G-CSFR expression on pre-radiation biopsy and tumor differentiation. This indicated that the G-CSF/G-CSFR signaling pathway may be involved in the development of rectal cancer, and that G-CSFR levels may be correlated with either aggressive disease or a poor prognosis. G-CSFR may mediate a cytoprotective response to reduce cell sensitivity to radiation, similar to the mechanism of accelerated proliferation in hematopoietic tumor cells. Therefore, preoperative radiotherapy may be able to eradicate G-CSFR$^-$ tumor cells, whereas G-CSFR$^+$ tumor cells may remain active because of their radio-resistance.

In conclusion, our study indicates that there is a significant correlation between G-CSFR expression and tumor radiosensitivity. Examination of G-CSFR expression suggested that pre-radiation biopsy specimens could predict the radiosensitivity of locally advanced rectal cancer before pre-operative irradiation, and that G-CSFR expression was associated with a lack of pathological complete response. Overall, our analysis of G-CSFR expression is helpful in determining a subgroup of high-risk patients suitable for more therapeutic modalities, such as appropriate monoclonal antibodies against G-CSFR.

COMMENTS

Background

Colorectal cancer is one of the most common cancers in humans. The expression of granulocyte colony-stimulating factor receptor (G-CSFR) is involved in the development of human colorectal cancer, and may be associated with more aggressive cancer. The authors investigated whether G-CSFR expression could predict tumor response or sphincter preservation in patients with rectal cancer receiving preoperative radiotherapy.

Research frontiers

Markers, including p53, transforming growth factor beta1 and epidermal growth factor receptor, have been studied in cell cycle regulation, apoptosis and resistance to radiation. Previous studies have proved that G-CSFR expression was upregulated in colorectal cancer cells, compared to the normal mucous membrane.

Innovations and breakthroughs

This study is the first to examine the relationship between G-CSFR expression and tumor radiosensitivity in rectal cancer. It was found that the expression of G-CSFR before preoperative irradiation may predict the radiosensitivity of rectal cancer. The current findings further reinforce their understanding of the mechanistic role of the G-CSF/G-CSFR signal in colorectal cancer.

Applications

G-CSFR expression in pre-radiation biopsy specimens can predict the radiosensitivity of locally advanced rectal cancer before preoperative irradiation. Analysis of G-CSFR expression is helpful in determining a subgroup of high-risk patients suitable for more therapeutic modalities, such as appropriate monoclonal antibodies against G-CSFR.

Terminology

G-CSFR, also known as cluster of differentiation 114, is a cell-surface receptor for granulocyte colony-stimulating factor and comprises an extracellular ligand-binding portion, a transmembrane domain and a cytoplasmic portion that is responsible for signal transduction.

Peer review

The authors evaluated the correlation between clinical or pathological parameters and the G-CSFR expression of tumors. The results presented were very interesting and suggest that the expression of G-CSFR before preoperative irradiation may predict radiosensitivity of rectal cancer.

REFERENCES

1 **Walker JJ**, Brewster DH, Colhoun HM, Fischbacher CM, Lindsay RS, Wild SH. Cause-specific mortality in Scottish patients with colorectal cancer with and without type 2 diabetes (2000-2007). *Diabetologia* 2013; **56**: 1531-1541 [PMID: 23624531 DOI: 10.1007/s00125-013-2917-x]

2 **Liu L**, Lemmens VE, De Hingh IH, de Vries E, Roukema JA, van Leerdam ME, Coebergh JW, Soerjomataram I. Second primary cancers in subsites of colon and rectum in patients with previous colorectal cancer. *Dis Colon Rectum* 2013; **56**: 158-168 [PMID: 23303143 DOI: 10.1097/DCR.0b013e318279eb30]

3 **Wilkinson N**, Scott-Conner CE. Surgical therapy for colorectal adenocarcinoma. *Gastroenterol Clin North Am* 2008; **37**: 253-267, ix [PMID: 18313549 DOI: 10.1016/j.gtc.2007.12.012]

4 **Penopoulos V**, Handolias M, Avgerinos A, Maris T, Ilias T, Issopoulos N, Christianopoulos G, Betsis D, Vrettou E, Kitis G, Pistevou K. A short course of preoperative radiotherapy improves prognosis of operable rectal carcinoma: a case control study. *Hepatogastroenterology* 2008; **55**: 1280-1287 [PMID: 18795673]

5 **Park IJ**, Kim HC, Yu CS, Kim TW, Jang SJ, Kim JC. Effect of adjuvant radiotherapy on local recurrence in stage II rectal cancer. *Ann Surg Oncol* 2008; **15**: 519-525 [PMID: 17960464 DOI: 10.1245/s10434-007-9643-x]

6 **Yoon SM**, Kim DY, Kim TH, Jung KH, Chang HJ, Koom WS, Lim SB, Choi HS, Jeong SY, Park JG. Clinical parameters predicting pathologic tumor response after preoperative chemoradiotherapy for rectal cancer. *Int J Radiat Oncol Biol Phys* 2007; **69**: 1167-1172 [PMID: 17967307 DOI: 10.1016/j.ijrobp.2007.04.047]

7 **Gosens MJ**, Dresen RC, Rutten HJ, Nieuwenhuijzen GA, van der Laak JA, Martijn H, Tan-Go I, Nagtegaal ID, van den Brule AJ, van Krieken JH. Preoperative radiochemotherapy is successful also in patients with locally advanced rectal cancer who have intrinsically high apoptotic tumours. *Ann Oncol* 2008; **19**: 2026-2032 [PMID: 18664561 DOI: 10.1093/annonc/mdn428]

8 **Yang X**, Liu F, Xu Z, Chen C, Wu X, Li G, Li J. Expression of granulocyte colony stimulating factor receptor in human colorectal cancer. *Postgrad Med J* 2005; **81**: 333-337 [PMID: 15879049 DOI: 10.1136/pgmj.2004.024646]

9 **Mandard AM**, Dalibard F, Mandard JC, Marnay J, Henry-Amar M, Petiot JF, Roussel A, Jacob JH, Segol P, Samama G. Pathologic assessment of tumor regression after preoperative chemoradiotherapy of esophageal carcinoma. Clinicopathologic correlations. *Cancer* 1994; **73**: 2680-2686 [PMID: 8194005]

10 **Wang J**, Yi J. Cancer cell killing via ROS: to increase or decrease, that is the question. *Cancer Biol Ther* 2008; **7**: 1875-1884 [PMID: 18981733]

11 **Rajkumar T**, Samson M, Rama R, Sridevi V, Mahji U, Swaminathan R, Nancy NK. TGFbeta1 (Leu10Pro), p53 (Arg72Pro) can predict for increased risk for breast cancer in south Indian women and TGFbeta1 Pro (Leu10Pro) allele predicts response to neo-adjuvant chemo-radiotherapy. *Breast Cancer Res Treat* 2008; **112**: 81-87 [PMID: 18058229 DOI: 10.1007/s10549-007-9821-3]

12 **Ahmed WA**, Suzuki K, Imaeda Y, Horibe Y. Ki-67, p53 and epidermal growth factor receptor expression in early glottic cancer involving the anterior commissure treated with radiotherapy. *Auris Nasus Larynx* 2008; **35**: 213-219 [PMID: 17996416 DOI: 10.1016/j.anl.2007.08.011]

13 **Quentmeier H**, Zaborski M, Drexler HG. The human bladder carcinoma cell line 5637 constitutively secretes functional cytokines. *Leuk Res* 1997; **21**: 343-350 [PMID: 9150352]

14 **Kikuchi T**, Nakahara S, Abe T. Granulocyte colony-stimulating factor (G-CSF) production by astrocytoma cells and its effect on tumor growth. *J Neurooncol* 1996; **27**: 31-38 [PMID: 8699223]

15 **Sarkar CA**, Lauffenburger DA. Cell-level pharmacokinetic model of granulocyte colony-stimulating factor: implications for ligand lifetime and potency in vivo. *Mol Pharmacol* 2003; **63**: 147-158 [PMID: 12488547]

P- Reviewers: Crea F, De Nardi P, Leitman M, Langner C, Hiraki M, Kim YJ, Triantafyllou K **S- Editor**: Gou SX **L- Editor**: Stewart GJ **E- Editor**: Wang CH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.1079

World J Gastroenterol 2014 January 28; 20(4): 1079-1087
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Role of hydrogen sulfide in portal hypertension and esophagogastric junction vascular disease

Chao Wang, Juan Han, Liang Xiao, Chang-E Jin, Dong-Jian Li, Zhen Yang

Chao Wang, Dong-Jian Li, Zhen Yang, Department of General Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China
Juan Han, Department of Biliary and Pancreatic Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China
Liang Xiao, Department of Surgery and Biological Therapy, Shenzhen Second People's Hospital, the First Affiliated Hospital of Shenzhen University, Shenzhen 518035, Guangdong Province, China
Chang-E Jin, Department of Respiratory Medicine, Shenzhen People's Hospital, Shenzhen 518020, Guangdong Province, China
Author contributions: Wang C, Han J and Xiao L designed the research; Wang C, Han J, Xiao L, Jin CE, Li DJ and Yang Z performed the research; Wang C, Han J, Xiao L and Jin CE analyzed the data; Wang C, Han J and Xiao L wrote the paper.
Supported by Specialized Research Fund for the Doctoral Program of Higher Education of China, No. 20120142120048; and Natural Science Foundation of Hubei Province, China, No. 2012FFB02308
Correspondence to: Chao Wang, PhD, Department of General Surgery, Tongji Hospital, Tongji Medical College, Science and Technology of Huazhong University, No. 1095, Jiefang Avenue, Wuhan 430030, Hubei Province, China. wangchao75@sina.com
Telephone: +86-27-83663008 Fax: +86-27-83663039
Received: August 16, 2013 Revised: October 14, 2013
Accepted: November 18, 2013
Published online: January 28, 2014

Abstract

AIM: To investigate the association between endogenous hydrogen sulfide (H_2S) and portal hypertension as well as its effect on vascular smooth muscle cells.

METHODS: Portal hypertension patients were categorized by Child-Pugh score based on bilirubin and albumin levels, prothrombin time, ascites and hepatic encephalopathy. Plasma H_2S concentrations and portal vein diameters (PVDs) were compared between portal hypertension patients and control participants, as well as between portal hypertension patients with varying degrees of severity. In addition, we established a rabbit hepatic schistosomiasis portal hypertension (SPH) model and analyzed liver morphology, fibrosis grade, plasma and liver tissue H_2S concentrations, as well as cystathionine γ-lyase (CSE) activity and phosphorylated extracellular signal-regulated kinase (pERK)1/2, B cell lymphoma (Bcl)-2 and Bcl-XL expression in portal vein smooth muscle cells, in addition to their H_2S-induced apoptosis rates.

RESULTS: In portal hypertension patients, endogenous H_2S levels were significantly lower than those in healthy controls. The more severe the disease was, the lower were the H_2S plasma levels, which were inversely correlated with PVD and Child-Pugh score. Liver tissue H_2S concentrations and CSE expression were significantly lower in the SPH rabbit livers compared with the control animals, starting at 3 wk, whereas pERK 1/2 expressions gradually increased 12-20 wk after SPH model establishment. In portal vein smooth muscle cells, increasing H_2S levels led to increased apoptosis, while Bcl-2 and Bcl-XL expression decreased.

CONCLUSION: H_2S prevents vascular restructuring caused by excessive proliferation of smooth muscle cells via apoptosis induction, which helps to maintain normal vascular structures.

Key words: Portal hypertension; Apoptosis; B-cell lymphoma-2; B-cell lymphoma-XL; Cystathionine γ-lyase; pERK 1/2; Hydrogen sulfide

Core tip: In portal hypertension patients, endogenous hydrogen sulfide (H_2S) levels were significantly lower than those in healthy controls. H_2S plasma level reductions correlated with portal vein diameter and Child-

Pugh score. In a rabbit hepatic schistosomiasis portal hypertension model, liver tissue H_2S concentrations and cystathionine γ-lyase expression were significantly reduced and phosphorylated extracellular signal regulated kinase 1/2 expression gradually increased. Increasing H_2S levels led to increased apoptosis of portal vein smooth muscle cells, while B-cell lymphoma-2 (Bcl-2) and Bcl-XL expression decreased. We suggest that H_2S prevents portal hypertension though apoptosis induction of otherwise excessive proliferating smooth muscle cells.

Wang C, Han J, Xiao L, Jin CE, Li DJ, Yang Z. Role of hydrogen sulfide in portal hypertension and esophagogastric junction vascular disease. *World J Gastroenterol* 2014; 20(4): 1079-1087 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/1079.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.1079

INTRODUCTION

Cirrhosis is a common chronic liver disease with various causes, and in the late stage, the main symptoms are liver function impairment and portal vein hypertension, which eventually lead to esophagogastric junction vascular diseases. Major critical pathophysiological processes accompanying the progression of cirrhosis are structural changes in the liver tissue and changes in vasoactive substance activation[1]. Recent studies have shown that the gaseous molecule hydrogen sulfide (H_2S) has biological signaling characteristics similar to NO and CO, and is involved in pathophysiological processes leading to portal hypertension through multiple mechanisms. H_2S, following NO and CO, is the third discovered endogenous gas signaling molecule, while various studies since the 1990s have shown that endogenous H_2S exists in many mammalian tissues and organs[2-5], serving as vascular and digestive tract smooth muscles relaxant, affecting long-term potentiation (LTP) induction in the hippocampus[6], regulating the secretion of corticotrophin-releasing hormone (CRH) in the hypothalamus[7], inhibiting the proliferation of vascular smooth muscle cells[8], and inducing smooth muscle relaxation in the human corpora cavernosum[9]. In mammals, there are two pathways for endogenous H_2S production: enzymatic and non-enzymatic. In the enzymatic pathway, which is the major source of endogenous H_2S, it is produced by cysteine breakdown through the actions of cystathionine-B-synthase (CBS) and cystathionine-γ-lyase (CSE). In the non-enzymatic pathway, glucose undergoes glycolysis and then combines with sulfur in the blood, thereby producing a small amount of H_2S. CBS and CSE are expressed in a tissue-specific manner; hepatocytes express both enzymes, whereas hepatic stellate cells (HSCs) express only CSE, and sinusoidal endothelial cells express neither[10]. Endogenous H_2S participates in the regulation of smooth muscle relaxation, therefore, it has been shown that it plays an important role in the development of primary hypertension and in the regulation of cardiovascular system functions and structures. The CSE/H_2S pathway participates in the pathophysiological processes of cardiovascular diseases, such as hypoxic pulmonary arterial hypertension and high pulmonary blood flow, leading to pulmonary arterial hypertension[11]. H_2S is an important vasoactive substance, thus, increasing attention has focused on its role in the development of portal hypertension. A 2003 study by Poliakova *et al*[12] found that a mixture of gases containing H_2S could induce biochemical restructuring of rat livers. Both long-term exposure (> 2 wk) to a low dose and short-term exposure to a high dose of the H_2S-containing gaseous mixture could lead to reversible changes in the liver. Although there have been studies about H_2S involvement in cirrhosis-induced portal hypertension, detailed mechanisms remain unclear. Some researchers[13-15] have speculated that H_2S participates in portal hypertension through the following three pathways: (1) regulation of smooth muscle relaxation; (2) inhibition of vascular smooth muscle proliferation; and (3) induction of vascular smooth muscle apoptosis. In this study we collected data about endogenous H_2S levels and other clinical data from patients with portal hypertension and also established rabbit portal hypertension as well as primary portal vein smooth muscle cell models in order to study correlations between H_2S and portal hypertension and its mode of action.

MATERIALS AND METHODS

Inclusion and exclusion criteria

This study included 200 patients with cirrhosis-induced portal hypertension who were treated in the Department of General Surgery of the Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, from December 2008 to December 2011. Clinical data and blood samples were collected from all 200 patients (140 males and 60 females, mean age 44 ± 14 years) and pathological studies identified 77 cases of hepatitis-induced cirrhosis, 82 of schistosomiasis-induced cirrhosis, 32 of cirrhosis of mixed causes, two of Caroli disease, four of portal vein cavernous transformation, and three of alcohol-induced cirrhosis. The control group comprised 100 healthy individuals examined at Tongji Hospital during the same period, consisting of 50 men and 50 women aged 47 ± 13 years. Exclusion criteria were chronic illnesses such as hypertension and pulmonary arterial hypertension, which could affect endogenous H_2S levels. Diagnosis of portal hypertension was made according to published guidelines[16] and portal vein diameter (PVD) was determined by Doppler ultrasound; if PVD was ⩾ 1.3 cm and accompanied with cirrhosis, the patient was diagnosed with portal hypertension. Child-Pugh scores were evaluated based on bilirubin and albumin levels, prothrombin time, and the presence of ascites and hepatic encephalopathy. All

participants gave their written informed consent and the study was approved by the Ethical Committee of the Tongji Hospital.

H_2S detection

Upon admission, 3 mL venous blood was collected from all participants in both groups, stored in sealed tubes for 2 h, and centrifuged at 3000 r/min for 10 min to separate the plasma and stored again at -70 ℃ in eppendorf (EP) tubes (Eppendorf, Hamburg, Germany). Plasma and liver tissue H_2S contents were measured according to Chunyu *et al*[17]. The resultant supernatant was analyzed using a UV spectrophotometer at 665 nm and calculated with a standard curve obtained from NaHS solutions. Plasma H_2S concentrations were expressed in μmol/L and liver tissue H_2S contents were shown in nmol/mg wet tissue/min. All measurements were performed in triplicate and results were expressed as mean ± SD.

Animal experiments

Thirty healthy adult long-eared white rabbits (purchased from Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China) with a body weight of 2.5-3.5 kg, regardless of sex, were randomly divided into a control (n = 10) and model (n = 20) group.

Model establishment

The animal model was established in the Schistosomiasis Research Facility, Hubei Provincial Disease Prevention and Control Center. Abdominal patches were used to infect the rabbits with schistosomal cercariae to induce a rabbit SPH model. The *Schistosoma japonicum* cercariae were collected from snails in Hubei Province. A 2 cm × 2 cm patch was collected into the abdominal region of the rabbits, creating a wound (Figure 1A). During wound cleansing, the cercariae were counted under a microscope (180 ± 10/rabbit) and the coverslips with the cercariae were located on the exposed skin for 15-20 min, leading to acute schistosomiasis infection. After infection, the animals were kept in rabbit rooms in the Tongji Hospital Laboratory Animal Facility. The control rabbits were treated similarly, apart from not being infected with the cercariae.

Sample collection

At 8, 12 and 16 wk after infection, five or six animals from the model group and three from the control group were randomly selected and peripheral venous blood samples were collected from the ear vein. The animals were sacrificed using air embolism; after which, portal vein blood was drawn and samples from liver and spleen tissues as well as portal vein blood vessels were taken. The tissue samples were stored in liquid nitrogen, while the blood samples were stored in a freezer.

Measurement of CSE activity in liver tissues

Using the method reported by Stipanuk *et al*[18], CSE activity was determined in a 1.0-mL volume by incubation of 100 mmol/L potassium phosphate buffer, pH 7.5, 4.0 mmol/L L-cystathionine, 0.125 nmol/L pyridoxal 5'-phosphate, 0.32 nmol/L NADH, 1.5 U lactate dehydrogenase, and 25 or 50 μL of the 20000 *g* supernatant prepared from liver homogenate in a 1.5 mL semi-micro quartz cuvette (10 mm light path) at 37 ℃. The decrease in OD_{340} gave a linear response to decreasing NADH concentrations, corresponding to lactate synthesis via L-cysteine-derived pyruvate accumulation and lactate dehydrogenase activity.

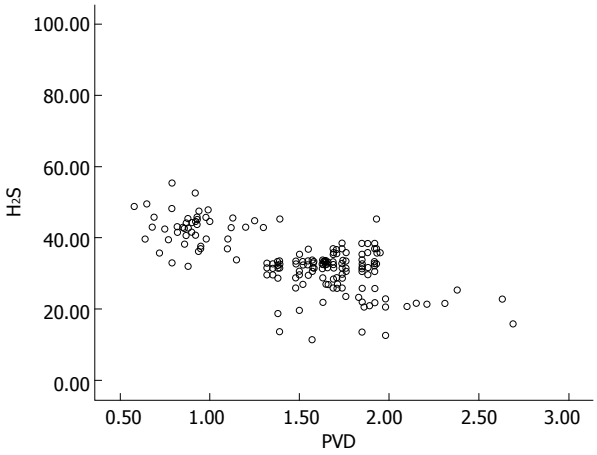


Figure 1 Negative correlation between H_2S plasma levels and portal vein diameters in patients with portal hypertension. r = -0.478, P < 0.05.

Liver hematoxylin and eosin staining

The rabbit liver tissues were fixed in a 10% formalin solution, embedded in paraffin, cut into 4-μm slices, stained with hematoxylin and eosin (HE), and sealed with neutral resin. Liver histology was observed under a microscope.

Masson's trichrome staining of liver tissues

The rabbit liver tissues were fixed in a 10% neutral formalin solution, embedded in paraffin, sliced, stained with potassium dichromate, acid fuchsin and aniline blue, and sealed with neutral resin. Morphological changes in the liver tissue were observed under an optical microscope. Collagen fibers were stained blue-green, muscle fibers and cellulose were stained red, and nuclei were stained blue to black.

Cell culture

Portal vein smooth muscle cells were grown in complete culture medium at 37 ℃ for 1-2 d and cell growth was monitored under a microscope. Cells were replated at 80%-90% confluence. Culture medium was changed on the basis of the actual cell growth.

Immunofluorescence and tissue cell apoptosis staining assays

Portal vein smooth muscle cells were inoculated on glass slides after conventional digestion. On the next day, the

Table 1 Comparison of age, liver enzymes and plasma H_2S levels between portal hypertension patients and healthy controls

	Portal hypertension group (n = 200)	Control group (n = 100)
Age (yr)	43.6 ± 14.4	47.1 ± 12.6
Albumin (g/dL)	33.0 ± 3.7	38.4 ± 4.1
TB (μmol/L)	31 ± 24	14 ± 13
ALT (U/L)	37 ± 29	25 ± 26
PVD (cm)	1.5 ± 0.4[a]	1.1 ± 0.3
H_2S		43.5 ± 6.2
Child-Pugh score		
A (H_2S) n = 48	42.6 ± 4.7[a]	
B (H_2S) n = 125	33.5 ± 7.7[b,c]	
C (H_2S) n = 27	22.2 ± 7.9[c,d,f]	

Significantly different compared with the control group, [a]$P < 0.05$, [b]$P < 0.01$; Compared with Child-Pugh score A group, both Child-Pugh score B and C groups had significantly lower values, [c]$P < 0.05$, [d]$P < 0.01$; Compared with the Child-Pugh score B group, the Child-Pugh score C group had a significantly lower value, [f]$P < 0.01$. TB: Tris-buffered; ALT: Alanine aminotransferase; PVD: Portal vein diameter.

cells were washed gently with PBS and 3.7% formaldehyde solution was added for 30 min at room temperature, after which they were washed three times with PBS for 2 min each. The cells were permeated for 10 min with 0.1% Triton-X-100 solution (prepared in PBS) and blocked with 10% goat serum (prepared in PBS) for 30 min, followed by incubation with rat α-smooth muscle actin (SMA) antibody (1:200 in PBS) at room temperature for 2 h. After washing three times with PBS, for 5 min each, the cells were incubated with fluorescent secondary antibody (1:200 dilute by PBS; Sigma, Shanghai, China) at room temperature in darkness for 1 h, followed by three times with PBS for 5 min each. Finally, we detected the fluorescence intensity using a fluorescence microscope (440/510 nm, excitation/emission) after adding fluorescent mounting medium.

Flow cytometry and apoptosis assay

Cultured portal vein smooth muscle cells at 80% confluence were harvested from 6-cm dishes, washed with PBS, and collected into 5 mL centrifuge tubes in triplicate. Cells (1×10^6) were prepared for apoptosis assays with an Annexin V/FITC Apoptosis Dectection Kit, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, United States) and then analyzed by flow cytometry.

Western blotting

Rabbit portal vein-tunica media dissections were prepared for detecting phosphorylated extracellular signal-regulated kinase (pERK)1/2 expression levels, and primary portal vein smooth muscle cells were used for analyzing B-cell lymphoma-XL (Bcl-XL) and Bcl-2, as well as β-actin expression. Protein concentrations were equalized after total protein extraction with lysate buffer and loading buffer was added, and samples were boiled and stored at -20 ℃ for further use. Ten microliters of the samples were subjected to SDS-PAGE and the protein was transferred to polyvinylidene difluoride membranes via wet electroblotting (21 V, 40 min). After 2 h blocking at room temperature in 5% bovine serum albumin, primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, United States) for Bcl-XL (1:400), Bcl-2 (1:400) and pERK1/2 (1:500) were added and the membranes were incubated for 2 h at room temperature and kept at 4 ℃ overnight. Samples were washed in Tris-buffered saline-Tween 20 (TBST), incubated with horseradish-peroxidase-conjugated secondary antibodies at room temperature for 90 min, and washed again with TBST. The membranes were placed in an imaging system to measure the signal intensities using chemiluminescent reagents.

Electron microscopy

Rabbit liver samples were cut into 1 mm^3 pieces, fixed in 2.5% phosphate-buffered glutaraldehyde for 2 h, and washed in phosphate buffer for 30 min. Samples were fixed in osmium tetroxide for 2 h and then washed in phosphate buffer for 30 min. Samples were incubated in 50% ethanol for 15 min, 70% ethanol for 40 min, 90% ethanol for 15 min, 90% acetone/90% ethanol (1:1) for 15 min, 90% acetone for 15 min, 100% acetone for 3 × 10 min, 100% acetone/embedding medium (1:1) overnight, pure embedding medium for 5 h, and embedded overnight. Sample slices were stained with toluidine blue and cut into ultrathin 100 nm slices. Samples were stained with uranyl acetate and lead nitrate and observed under a transmission electron microscope to observe ultrastructural pathological changes.

Statistical analysis

Data were analyzed using SPSS version 12.0 software. Quantitative variables were described using mean ± SD in the case of normal distribution and each sample was measured at least three times. Possible differences between groups were evaluated using analysis of variance (ANOVA) in the case of continuous data. In the case of overall significant differences as a result of ANOVA, pair-wise t tests were conducted using the closed testing procedure. Analysis of PVD and endogenous H_2S concentrations was done by linear correlation. Statistical significance was considered if $P < 0.05$.

RESULTS

Plasma H_2S concentration in portal hypertension patients and controls

There was no significant difference in age between the control and portal hypertension groups. However, in the portal hypertension group, endogenous plasma H_2S levels were significantly lower than those in the control group, correlating with disease severity. Furthermore, plasma H_2S concentrations correlated inversely with PVD (r = -0.478, $P < 0.05$) (Table 1, Figure 1).

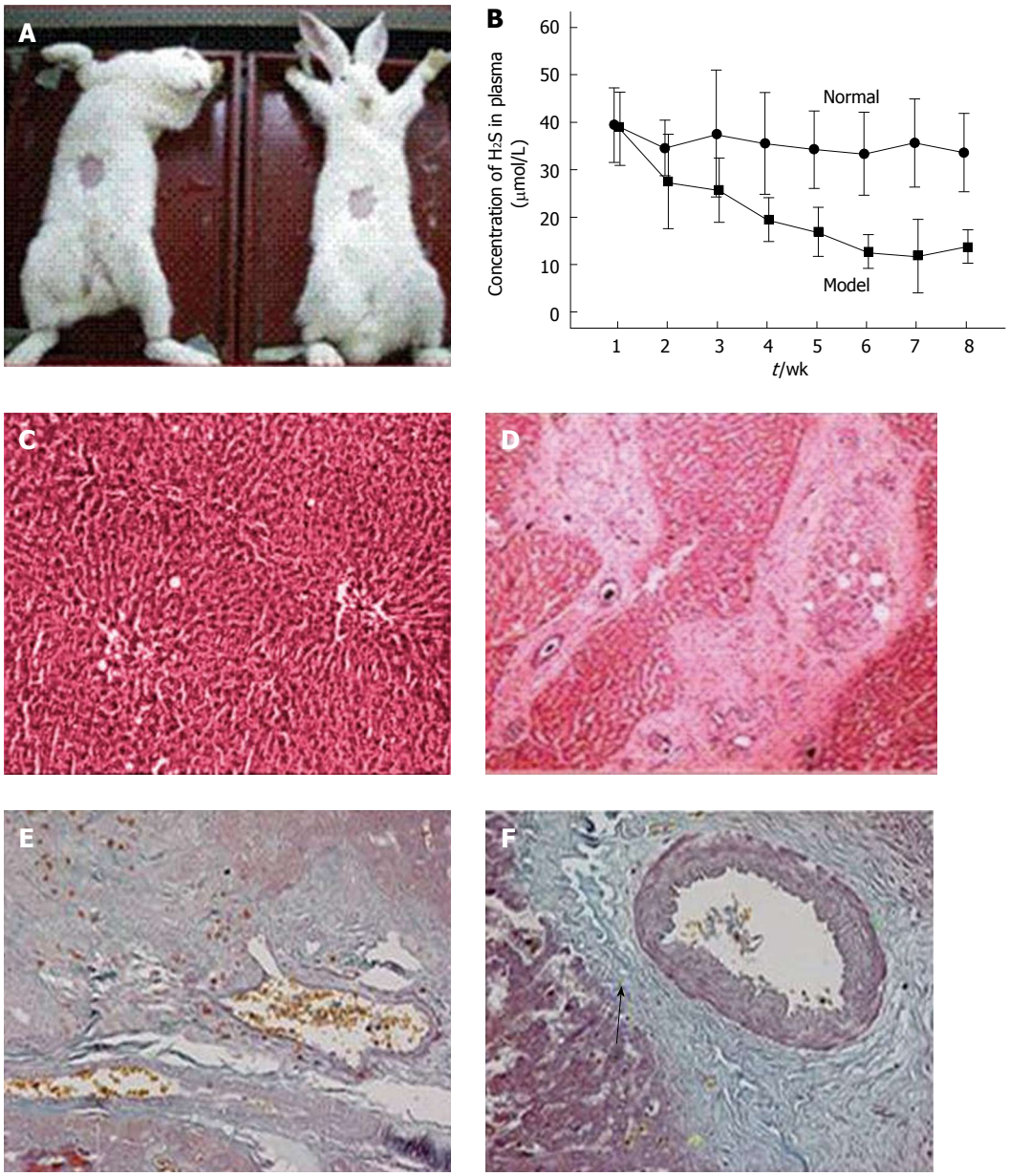


Figure 2 Liver hematoxylin and eosin and Masson's trichrome staining of rabbits in portal hypertension model group and control group. A: Rabbit portal hypertension model; B: Relationship between rabbit portal hypertension progression and H_2S concentration; C: Normal rabbit liver tissue [hematoxylin and eosin (HE) staining]; D: Schistosomiasis portal hypertension (SPH) rabbit liver sample with concentric arrangement of fibrous larval nodules and fibrous connective tissue in the portal area (HE staining); E and F: Masson's trichrome staining: collagen fibers are stained blue-green; muscle fibers and cellulose are stained red; and nuclei are stained blue to black; E: Normal liver tissue; F: SPH liver tissue with large amount of collagen fibers (arrow). All histological images are shown with × 40 magnification.

Liver morphology and H_2S changes in the rabbit hepatic SPH model

During the progression of schistosomiasis-induced cirrhosis in rabbits, plasma H_2S concentrations showed a clearly descending trend and starting from week 3, there were significant differences compared to the control group (Figure 2B). Furthermore, HE staining of the SPH rabbit livers showed that the liver hepatocytes of the control group were neatly arranged with no significant proliferation of fibrous tissue in the portal areas and lobular structures were maintained (Figure 2C). In contrast, in the portal areas of the SPH rabbit livers, fibrous larval nodules were arranged in concentric rings and there were also significant proliferation and widening of the fibrous connective tissues in the portal areas with hyaline appearance (Figure 2D). There was a small amount of collagen fibers in the central venous tissue of normal livers (Figure 2E), whereas in the SPH rabbit livers, a larger amount of collagen fibers stained blue-green (arrow) (Figure 2F).

H_2S and CSE concentrations in SPH rabbit and control liver tissues

CSE activity and H_2S concentrations were both lower in the SPH rabbit livers (Table 2). In order to elucidate whether the H_2S-related differences had an effect on

Table 2 H_2S concentration and cystathionine γ-lyase activity in rabbit liver tissues

		Control	SPH
H_2S concentration (nmol/mg wet tissue/min)	8W	413.66 ± 57.38	326.43 ± 33.67[a]
	12W	435.31 ± 442.46	236.57 ± 26.39[a]
	16W	420.79 ± 31.52	158.64 ± 41.77[a]
CSE (mmol/mg per min)	8W	120.64 ± 9.37	114.71 ± 11.34
	12W	178.23 ± 19.32	263.32 ± 28.97[a]
	16W	118.35 ± 9.82	82.67 ± 7.48[a]

Statistically significant differences compared with the control group, [a]P < 0.05. SPH: Schistosomiasis portal hypertension; CSE: Cystathionine γ-lyase.

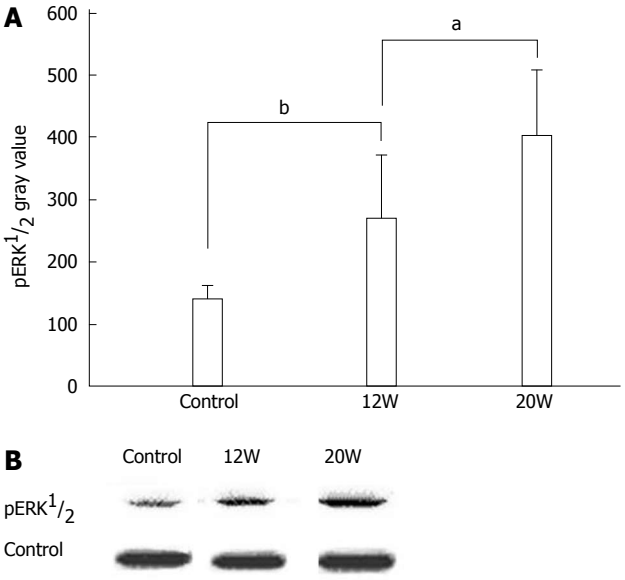


Figure 3 Western blotting analysis of phosphorylated extracellular signal-regulated kinase 1/2 expression levels in schistosomiasis portal hypertension and control rabbit portal vein-tunica media dissections. [a]P < 0.05,[b]P < 0.001.

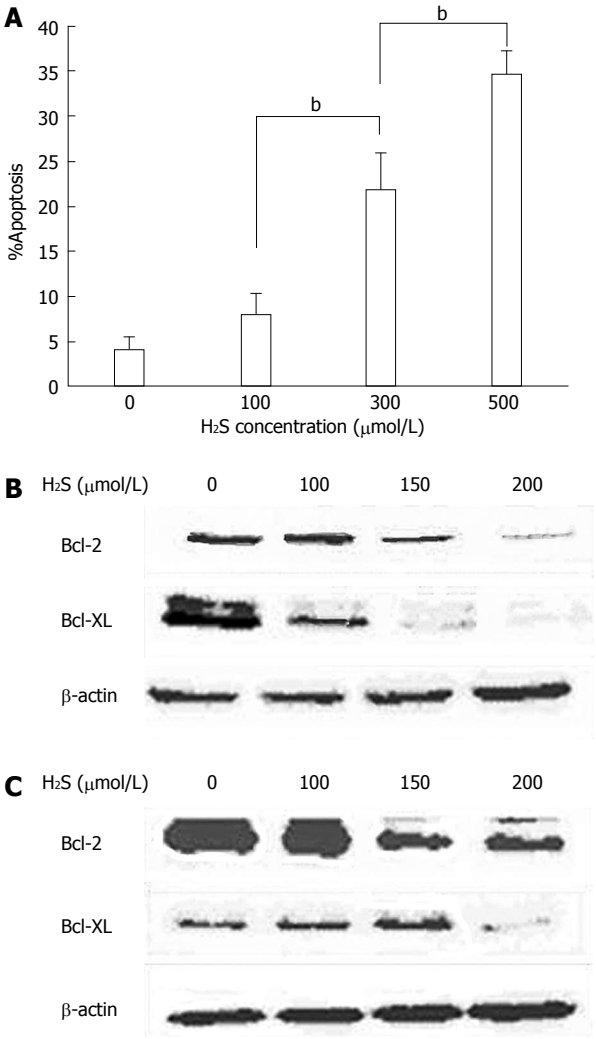


Figure 4 (A) Smooth muscle cell apoptosis under varying H_2S concentrations, observed by flow cytometry, (B) Protein levels of the apoptotic factors Bcl-2 and Bcl-XL in primary rabbit portal vein smooth muscle cells and (C) in primary human portal vein smooth muscle cells. [b]P < 0.001.

cell proliferation-related proteins, we measured pERK 1/2 expression levels in dissected SPH rabbit liver portal vein-tunica intima tissues and found that pERK 1/2 protein levels increased significantly with disease progression in the rabbit portal hypertension model (Figure 3).

H_2S inhibits anti-apoptotic Bcl-2 and Bcl-XL gene expression and triggers apoptosis in healthy liver cells

We collected primary rabbit portal vein smooth muscle cells and after cultivation, added H_2S to the medium to observe its effect on apoptosis and Bcl-2 and Bcl-XL expression. Flow cytometry revealed that, with increasing concentrations of H_2S in the cell culture medium, the apoptosis rate of the portal vein smooth muscle cells also increased (Figure 4A). In addition, with increasing H_2S concentration, there was also decreased expression of the anti-apoptotic genes Bcl-2 and Bcl-XL (Figure 4B and C).

Our immunofluorescence apoptosis assay showed that vascular smooth muscle cells of the omentum underwent significant apoptosis under normal H_2S concentrations (50 μmol.L), which was reduced without H_2S (Figure 5A and B). Under the electron microscope, we observed that with 50 μmol/L H_2S in the medium, the nuclear membrane in some cells disintegrated, the nucleoplasm condensed, and the cells clearly underwent irreversible cell death. In contrast, when smooth muscle cells were cultured without H_2S, the nucleus was round or had minor indentations, there was abundant evenly distributed euchromatin and the nucleolus was clearly visible. The mitochondria were round or kidney-shaped and the overall structural integrity was maintained (Figure 5C and D). These results indicated that a certain concentration of H_2S leads to vascular smooth muscle cell apoptosis.

DISCUSSION

Portal hypertension is one of the two most serious conditions resulting from cirrhosis. Signs include open-

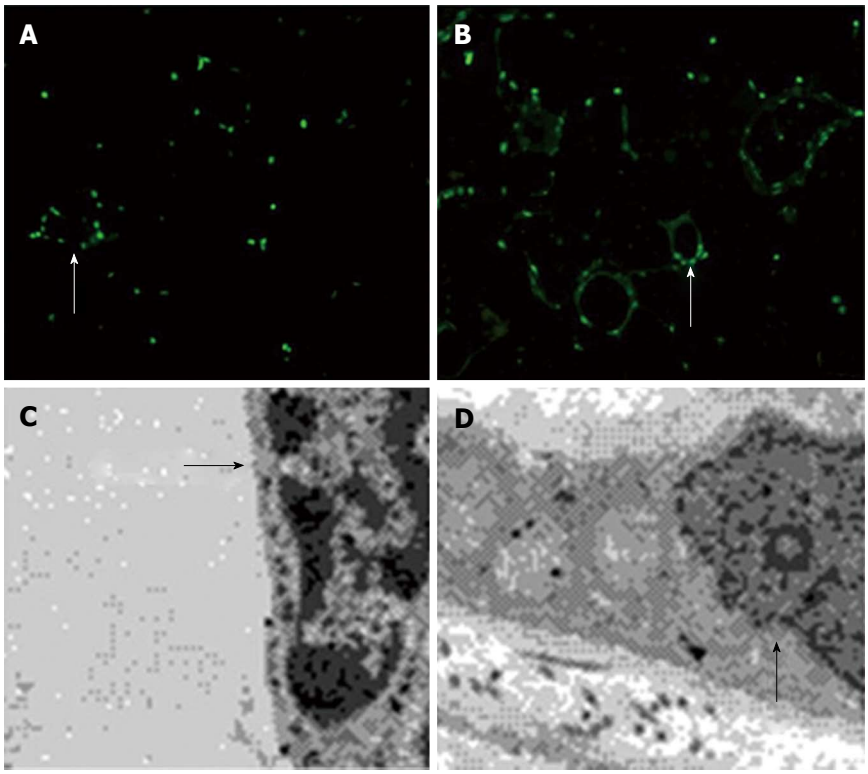


Figure 5 Effect of H_2S concentrations on portal vein smooth muscle cell apoptosis rates. A: Immunofluorescence assays showed significant apoptosis in normal rabbit omentum vascular smooth muscle cells under normal H_2S (50 μmol/L) concentration; B: There was significantly less apoptosis in the cells without H_2S (magnification × 40); C: Under an electron microscope, with normal H_2S concentrations, rupturing of the nuclear membrane and condensation of the nucleoplasm was observed, indicating irreversible cell death; D: In cells without H_2S substitution, the mitochondria appeared intact and the structural integrity was maintained.

ing of the systemic portal collateral circulation, splenomegaly, hypersplenism and ascites. Portal hypertension arises when liver fibrosis and regenerative nodules create pressure on the liver sinusoids and hepatic veins, thus increasing portal vein resistance. Impaired liver function and an imbalance of various vasoactive substances, which can result from multiple causes, are important factors in maintaining and exacerbating portal hypertension[19]. The main cause of increased resistance is that contraction of sinusoidal endothelial cells increases resistance in the liver sinusoids and contraction reactivity of intrahepatic blood vessels, leading to increased contractility in the portal venous system. Increased liver sinusoidal resistance is in turn dependent on HSC contraction. It is reported that portal hypertension can be caused by increased production of vasoconstrictors and decreased production of vasodilators. H_2S is an important vasodilator in the hepatic microcirculation and causes relaxation of vascular smooth muscles[20,21]. In a previous study, NaHS solutions were injected into the abdominal cavity of rats with cirrhosis for 5 d, and NaHS was perfused through the liver tissue outside the body. NaHS significantly relaxed vascular smooth muscles and reduced HSC contraction, thus reducing intrahepatic resistance[10]. These results showed to a certain extent that treatment with exogenous H_2S can reverse the excessive intrahepatic resistance caused by decreased production of endogenous H_2S in cirrhosis. Our study also showed in line with the previous findings that patients with portal hypertension had lower endogenous H_2S concentrations than healthy controls, and that the lower the concentration, the more severe was the disease, while plasma H_2S concentrations were inversely related with PVD. Our SPH results showed that rabbits with schistosomiasis-induced portal hypertension had lower plasma H_2S concentrations after 3 wk than the control group had, and CSE activity in their tissue declined. A previous article reported that reduced CSE expression in cirrhotic liver contributed to the development of increased intrahepatic resistance and portal hypertension[10]. Hepatocyte apoptosis plays an important role in normal liver development and in various liver diseases[22,23]. When HSCs proliferate, they produce extracellular matrix and collagen, which leads to liver fibrosis, and it has been reported that H_2S induces apoptosis[24] and inhibits HSC activation[25], which reduces vascular restructuring and aggravates hypertension. We found that H_2S led to reduced expression of antiapoptotic Bcl-2 and Bcl-XL proteins, in addition to elevated apoptosis.

In summary, we found that portal hypertension patients had significantly lower serum H_2S concentrations and that disease severity and PVD were correlated with H_2S concentration. In addition, our SPH model revealed that liver cirrhosis led to low serum and liver tissue H_2S concentrations, and reduced liver tissue CSE activity, while pERK1/2 expression gradually increased. In

conclusion, we suggest that H_2S deprivation may play a role in the initiation, progression and exacerbation of cirrhosis-related portal hypertension through reduction of portal vein smooth muscle cell apoptosis and concomitant pathological blood vessel restructuring.

ACKNOWLEDGMENTS

We specially thank Jian-Li Wu for her help with the project design.

COMMENTS

Background

Major critical pathophysiological processes of cirrhosis are structural changes in the liver tissues leading to portal hypertension.

Research frontiers

H_2S, following NO and CO, is the third discovered endogenous gas signaling molecule, serving as a relaxant of vascular and digestive tract smooth muscles, inhibiting the proliferation of vascular smooth muscle cells, and inducing smooth muscle relaxation in the human corpora cavernosum.

Innovations and breakthroughs

This study found that portal hypertension patients had significant lower serum H_2S concentrations and that the severity of the disease and portal vein diameter correlated with H_2S concentration. H_2S concentration and cystathionine γ-lyase (CSE) expression were significantly lower in schistosomiasis portal hypertension (SPH) rabbit livers, and phosphorylated extracellular signal-regulated kinase (pERK)1/2 expression was increased. In portal vein smooth muscle cells, increasing H_2S levels led to increased apoptosis, while B-cell lymphoma (Bcl)-2 and Bcl-XL expression decreased.

Applications

H_2S might be applied for treatment of liver cirrhosis.

Terminology

pERK1/2, after stimulation by mitogens, hormones or growth factors, is involved in cell growth and differentiation, whereas Bcl-2 and Bcl-XL are antiapoptotic genes. Without H_2S, high pERK1/2 and Bcl2 Bcl-XL activities lead to enhanced proliferation and reduced apoptosis rates in liver cells.

Peer review

In their manuscript the authors first describe their clinical observation, that there is a correlation between H_2S serum levels and portal vein diameter in portal hypertension patients. Then they extended their research on a rabbit hepatic schistosomiasis portal hypertension model and analyzed morphological changes and expression of the proliferation and apoptosis related genes. They concluded that H_2S might be an important signal molecule for the integrity of hepatic veins. The research is interesting and might lead to a new approach for treatment of portal hypertension.

REFERENCES

1 **Laleman W**. Role of vasoactive substances and cellular effectors in the pathophysiology of cirrhotic portal hypertension: the past, the present and the future--Georges Brohée Lecture. *Acta Gastroenterol Belg* 2009; **72**: 9-16 [PMID: 19402365]

2 **Gharib-Naseri MK**, Saberi S, Mard SA, Latifi SM. Bronchodilatory effect of hydrogen sulfide in rat. *Iran J Basic Med Sci* 2012; **15**: 907-915 [PMID: 23493917]

3 **Jung KJ**, Jang HS, Kim JI, Han SJ, Park JW, Park KM. Involvement of hydrogen sulfide and homocysteine transsulfuration pathway in the progression of kidney fibrosis after ureteral obstruction. *Biochim Biophys Acta* 2013; **1832**: 1989-1997 [PMID: 23846016 DOI: 10.1016/j.bbadis.2013.06.015]

4 **Pan LL**, Liu XH, Shen YQ, Wang NZ, Xu J, Wu D, Xiong QH, Deng HY, Huang GY, Zhu YZ. Inhibition of NADPH oxidase 4-related signaling by sodium hydrosulfide attenuates myocardial fibrotic response. *Int J Cardiol* 2013; **168**: 3770-3778 [PMID: 23830348 DOI: 10.1016/j.ijcard.2013.06.007]

5 **Hosoki R**, Matsuki N, Kimura H. The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. *Biochem Biophys Res Commun* 1997; **237**: 527-531 [PMID: 9299397 DOI: 10.1006/bbrc.1997.6878]

6 **Abe K**, Kimura H. The possible role of hydrogen sulfide as an endogenous neuromodulator. *J Neurosci* 1996; **16**: 1066-1071 [PMID: 8558235]

7 **Kimura H**. Hydrogen sulfide as a neuromodulator. *Mol Neurobiol* 2002; **26**: 13-19 [PMID: 12392053 DOI: 10.1385/MN:]

8 **Chen XB**, Du JB, Zhang CY, Tang CS, Zhou WJ. [Effect of hydrogen sulfide, a new gaseous signal molecule, on pulmonary vascular smooth muscle cell apoptosis in rats]. *Beijing Da Xue Xue Bao* 2004; **36**: 341-344 [PMID: 15303121]

9 **d'Emmanuele di Villa Bianca R**, Sorrentino R, Maffia P, Mirone V, Imbimbo C, Fusco F, De Palma R, Ignarro LJ, Cirino G. Hydrogen sulfide as a mediator of human corpus cavernosum smooth-muscle relaxation. *Proc Natl Acad Sci USA* 2009; **106**: 4513-4518 [PMID: 19255435 DOI: 10.1073/pnas.0807974105]

10 **Fiorucci S**, Antonelli E, Mencarelli A, Orlandi S, Renga B, Rizzo G, Distrutti E, Shah V, Morelli A. The third gas: H2S regulates perfusion pressure in both the isolated and perfused normal rat liver and in cirrhosis. *Hepatology* 2005; **42**: 539-548 [PMID: 16108046 DOI: 10.1002/hep.20817]

11 **Qingyou Z**, Junbao D, Weijin Z, Hui Y, Chaoshu T, Chunyu Z. Impact of hydrogen sulfide on carbon monoxide/heme oxygenase pathway in the pathogenesis of hypoxic pulmonary hypertension. *Biochem Biophys Res Commun* 2004; **317**: 30-37 [PMID: 15047144 DOI: 10.1016/j.bbrc.2004.02.176]

12 **Poliakova VS**, Shakhlamov VA, Stadnikov AA, Solnyshkova TG. [Structural-biochemical reorganization of rat liver caused by hydrogen sulfide-containing gas mixture]. *Morfologiia* 2003; **124**: 84-87 [PMID: 14628564]

13 **Ebrahimkhani MR**, Mani AR, Moore K. Hydrogen sulphide and the hyperdynamic circulation in cirrhosis: a hypothesis. *Gut* 2005; **54**: 1668-1671 [PMID: 16174660 DOI: 10.1136/gut.2004.056556]

14 **Köhn C**, Dubrovska G, Huang Y, Gollasch M. Hydrogen sulfide: potent regulator of vascular tone and stimulator of angiogenesis. *Int J Biomed Sci* 2012; **8**: 81-86 [PMID: 23675260]

15 **Schleifenbaum J**, Köhn C, Voblova N, Dubrovska G, Zavarirskaya O, Gloe T, Crean CS, Luft FC, Huang Y, Schubert R, Gollasch M. Systemic peripheral artery relaxation by KCNQ channel openers and hydrogen sulfide. *J Hypertens* 2010; **28**: 1875-1882 [PMID: 20577128 DOI: 10.1097/HJH.0b013e32833c20d5]

16 **Zhu YZ**. A Surgical Illustration of Portal Hypertension. Shenyang: Liaoning Science and Technology Press, 2006: 68-126

17 **Chunyu Z**, Junbao D, Dingfang B, Hui Y, Xiuying T, Chaoshu T. The regulatory effect of hydrogen sulfide on hypoxic pulmonary hypertension in rats. *Biochem Biophys Res Commun* 2003; **302**: 810-816 [PMID: 12646242 DOI: 10.1016/S0006-291X(03)00256-0]

18 **Stipanuk MH**, Beck PW. Characterization of the enzymic capacity for cysteine desulphhydration in liver and kidney of the rat. *Biochem J* 1982; **206**: 267-277 [PMID: 7150244]

19 **Suematsu M**, Ishimura Y. The heme oxygenase-carbon monoxide system: a regulator of hepatobiliary function. *Hepatology* 2000; **31**: 3-6 [PMID: 10613719 DOI: 10.1002/hep.510310102]

20 **Liu H**, Zheng Y, Chen W, Zhao J, Li R, Zhang N, Liu F, Yan J. Effect of endogenous hydrogen sulfide on apoptosis of

cirrhosis rat liver cells. *Shijie Huaren Xiaohua Zazhi* 2012; **20**: 670-674

21 **Liu Y**, Li Y, Yang W, Cao G. H2 S inhibits the activation of hepatic stellate cells and downregulates the expression of urotensin II. *Hepatol Res* 2013; **43**: 670-678 [PMID: 23131022 DOI: 10.1111/hepr.12002]

22 **Guicciardi ME**, Gores GJ. Apoptosis: a mechanism of acute and chronic liver injury. *Gut* 2005; **54**: 1024-1033 [PMID: 15951554 DOI: 10.1136/gut.2004.053850]

23 **Kiliçarslan A**, Kahraman A, Akkiz H, Yildiz Menziletoğlu S, Fingas CD, Gerken G, Canbay A. Apoptosis in selected liver diseases. *Turk J Gastroenterol* 2009; **20**: 171-179 [PMID: 19821198]

24 **Fan HN**, Wang HJ, Yang-Dan CR, Ren L, Wang C, Li YF, Deng Y. Protective effects of hydrogen sulfide on oxidative stress and fibrosis in hepatic stellate cells. *Mol Med Rep* 2013; **7**: 247-253 [PMID: 23129058]

25 **Lu F**, Xing J, Zhang X, Dong S, Zhao Y, Wang L, Li H, Yang F, Xu C, Zhang W. Exogenous hydrogen sulfide prevents cardiomyocyte apoptosis from cardiac hypertrophy induced by isoproterenol. *Mol Cell Biochem* 2013; **381**: 41-50 [PMID: 23660955 DOI: 10.1007/s11010-013-1686-7]

P- Reviewers: Gentilucci UV, Klinge U **S- Editor**: Qi Y
L- Editor: Wang TQ **E- Editor**: Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.1088

World J Gastroenterol 2014 January 28; 20(4): 1088-1094
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Diagnostic value of contrast enhanced ultrasound for splenic artery complications following acute pancreatitis

Di-Ming Cai, Shyam Sundar Parajuly, Wen-Wu Ling, Yong-Zhong Li, Yan Luo

Di-Ming Cai, Shyam Sundar Parajuly, Wen-Wu Ling, Yong-Zhong Li, Yan Luo, Department of Ultrasound, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Author contributions: Cai DM, Luo Y and Li YZ designed the study; Parajuly SS and Ling WW revised the manuscript; Cai DM wrote the manuscript.

Correspondence to: Dr. Yan Luo, Department of Ultrasound, West China Hospital, Sichuan University, No. 37, Guoxuexiang, Chengdu 610041, Sichuan Province, China. hxluoyan@126.com
Telephone: +86-28-85423192 Fax: +86-28-85422192
Received: August 22, 2013 Revised: November 1, 2013
Accepted: November 12, 2013
Published online: January 28, 2014

Abstract

AIM: To assess the value of contrast-enhanced ultrasound (CEUS) in diagnosing splenic artery complications (SACs) after acute pancreatitis (AP).

METHODS: One hundred and eighteen patients with AP were enrolled in the study. All patients were examined by CEUS and contrast-enhanced computed tomography (CECT). CECT was accepted as a gold standard for the diagnosis of SACs in AP. The diagnostic accuracy of splenic CEUS and pancreatic CEUS was compared with that of CECT. Splenic infarction was the diagnostic criterion for splenic artery embolism and local dysperfusion of the splenic parenchyma was the diagnostic criterion for splenic arterial stenosis. The incidence of splenic sub-capsular hemorrhage, splenic artery aneurysms, and splenic rupture was all lower than that of SACs.

RESULTS: Nine patients were diagnosed as having SACs after AP by CECT among the 118 patients. The patients with SACs were diagnosed with severe acute pancreatitis (SAP). Among them, 6 lesions were diagnosed as splenic artery embolism, 5 as splenic artery aneurysms, and 1 as splenic arterial stenosis. No lesion was diagnosed by pancreatic CEUS and 5 lesions were diagnosed by splenic CEUS. By splenic CEUS, 4 cases were diagnosed as splenic artery embolism and 1 as splenic arterial stenosis. The accuracy of splenic CEUS in diagnosis of SACs in SAP was 41.7% (5/12), which was higher than that of pancreatic CEUS (0%).

CONCLUSION: Splenic CEUS is a supplementary method for pancreatic CEUS in AP patients, which can decrease missed diagnosis of SACs.

Key words: Acute pancreatitis; Severe acute pancreatitis; Contrast enhanced ultrasound; Splenic artery complications; Splenic contrast-enhanced computed tomography

Core tip: We prospectively investigated splenic contrast-enhanced ultrasound (CEUS) in diagnosis of splenic artery complications in acute pancreatitis (AP). The diagnostic yield of splenic CEUS for detecting splenic artery complications (SACs) in AP was higher than that of pancreatic CEUS. Splenic CEUS is a supplementary method for pancreatic CEUS when an AP patient needs pancreatic CEUS examination, which can decrease missed diagnosis of SACs.

Cai DM, Parajuly SS, Ling WW, Li YZ, Luo Y. Diagnostic value of contrast enhanced ultrasound for splenic artery complications following acute pancreatitis. *World J Gastroenterol* 2014; 20(4): 1088-1094 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/1088.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.1088

INTRODUCTION

Contrast-enhanced ultrasound (CEUS) has become an

Table 1 Splenic lesions and splenic artery complications

Splenic lesions	Splenic artery complications
Splenic infarction/subcapsular hemorrhage	Splenic artery embolism
Local dysperfusion of splenic parenchyma	Splenic artery stenosis
Splenic artery aneurysms	Splenic artery aneurysms
Splenic rupture	Splenic artery embolism

important imaging method to evaluate the degree of severity and predict the prognosis of diseases[1]. Acute pancreatitis (AP) can be examined by CEUS to identify the necrosis range of the pancreas, which is related to treatment and prognosis. Splenic artery complications (SACs) have a low morbidity in AP. Although SACs are rare complications in AP and most patients present few clinical symptoms, it may lead to a severe outcome. Rupture of splenic artery aneurysms is frequent and sometimes occurs as the first symptom, and sometimes it is fatal[2]. So, it is of great clinical significance to determine the diagnostic accuracy of CEUS for SACs in AP. However, the sensitivity of gray scale ultrasound and color Doppler ultrasound is low, and few studies have reported on CEUS[3]. In this article, we discussed the diagnostic value and accuracy of splenic CEUS for SACs in AP.

MATERIALS AND METHODS

Patient data

This study was conducted with the approval of the Ethics Committee of West China Hospital and informed consent was obtained from all the patients. We collected 129 in-patients with AP treated at our hospital from March 2010 to March 2013. Eleven patients were excluded from the study either because the gray scale sonography of the pancreas hardly displayed meteorism or because patients had a history of splenic disease. Of the remaining 118 patients included in the study, there were 69 males and 49 females, with a mean age of 41.27 ± 12.15 years (range, 15-79). All patients were examined by pancreatic CEUS to diagnose the SACs in AP. Splenic CEUS was also used to diagnose the splenic lesions. All contrast-enhanced computed tomography (CECT) and CEUS examinations were performed at an interval of 72 h. The study protocol was kept blind for doctors performing CEUS and CECT.

Sonographic examination

A LOGIQ E9 (GE Healthcare, Milwaukee, WI, United States) ultrasound system with a C1-5 MHz probe or a PHILIPS IU22 (Philips Medical Systems, Bothell, WA, United States) ultrasound system with a C5-2 MHz transducer was selected. Both the ultrasound systems were equipped with harmonic contrast pulse sequencing apparatus. The contrast agent used was SonoVue (BraccoSpa, Milan, Italy) and the suspension contained stabilized sulfur hexafluoride microbubbles.

Two sonologists, who had over ten years of experience in abdominal conventional ultrasound and over three years of experience in CEUS evaluation for pancreatic diseases, performed the examinations. All patients were asked to fast before ultrasound examination. Firstly, gray scale sonography and color Doppler flow imaging were performed to observe the size and shape of the pancreas and spleen, peri-pancreatic fluid collection, echogenicity of the parenchyma, and the inner diameters of the splenic artery. Then, CEUS was started at a low mechanical index (GE MI: 0.12; PHILIPS MI: 0.06). SonoVue suspension of 1.5-2.0 mL was administered as a bolus injection through the antecubital vein, followed by a flush with 5 mL saline solution. Real-time contrast-enhanced imaging was recorded on the hard disk when the suspension (SonoVue) was injected, and the times were calculated simultaneously. Based on the entire arterial pancreatic or splenic system, the contrast phase was identified as the arterial phase (0-30 s after contrast agent injection) and the venous phase (starting at 31 s after contrast agent injection). When a sonologist finished pancreatic CEUS, the other sonologist started splenic CEUS successively. And the results of SACs by pancreatic CEUS and by splenic CEUS were recorded.

The splenic CEUS images for SACs were correlated with the pathophysiologic results (Table 1). The results of splenic lesions were transformed into corresponding SACs and recorded immediately.

Computer tomography

A 64-slice spiral computer tomography (CT) (Philips Brilliance; Philips Medical Systems, Cleveland, OH, United States) system or 16-slice spiral CT system (Somatom Sensation 16; Siemens, Erlangen, Germany) was used in this study. The contrast agent used was iopamidol (Iopamiro; Bracco Imaging) or iopromide (Ultravist; Bayer Schering, Germany) at a concentration of 37 gI/100 mL.

The range of CECT scanning was from the chest to the pelvic floor. Iopamidol or iopromide was injected with a total amount of 90-120 mL to each patient at a rate of 3 mL/s with a power injector through the antecubital vein.

RESULTS

The 118 patients were diagnosed with AP by CECT; among them only 9 patients (including 12 lesions) were diagnosed as having SACs. Among the 118 patients, there were 38 patients with mild AP and 80 with severe AP (SAP). All SACs patients were from the SAP group. Six cases were diagnosed as splenic artery embolism; 5 cases as splenic artery aneurysms and 1 case as splenic arterial stenosis by CECT. Three patients had splenic artery embolism with splenic artery aneurysms found by CECT. In the splenic CEUS group, SACs were found in 5 cases, including 4 cases of splenic artery embolism (Figure 1) and 1 case of splenic arterial stenosis (Figure 2). And in the pancreatic CEUS group, all lesions of SACs were misdiagnosed (Table 2). The diagnostic accuracy of splenic CEUS for SACs in AP was 41.7% (5/12), which was ob-

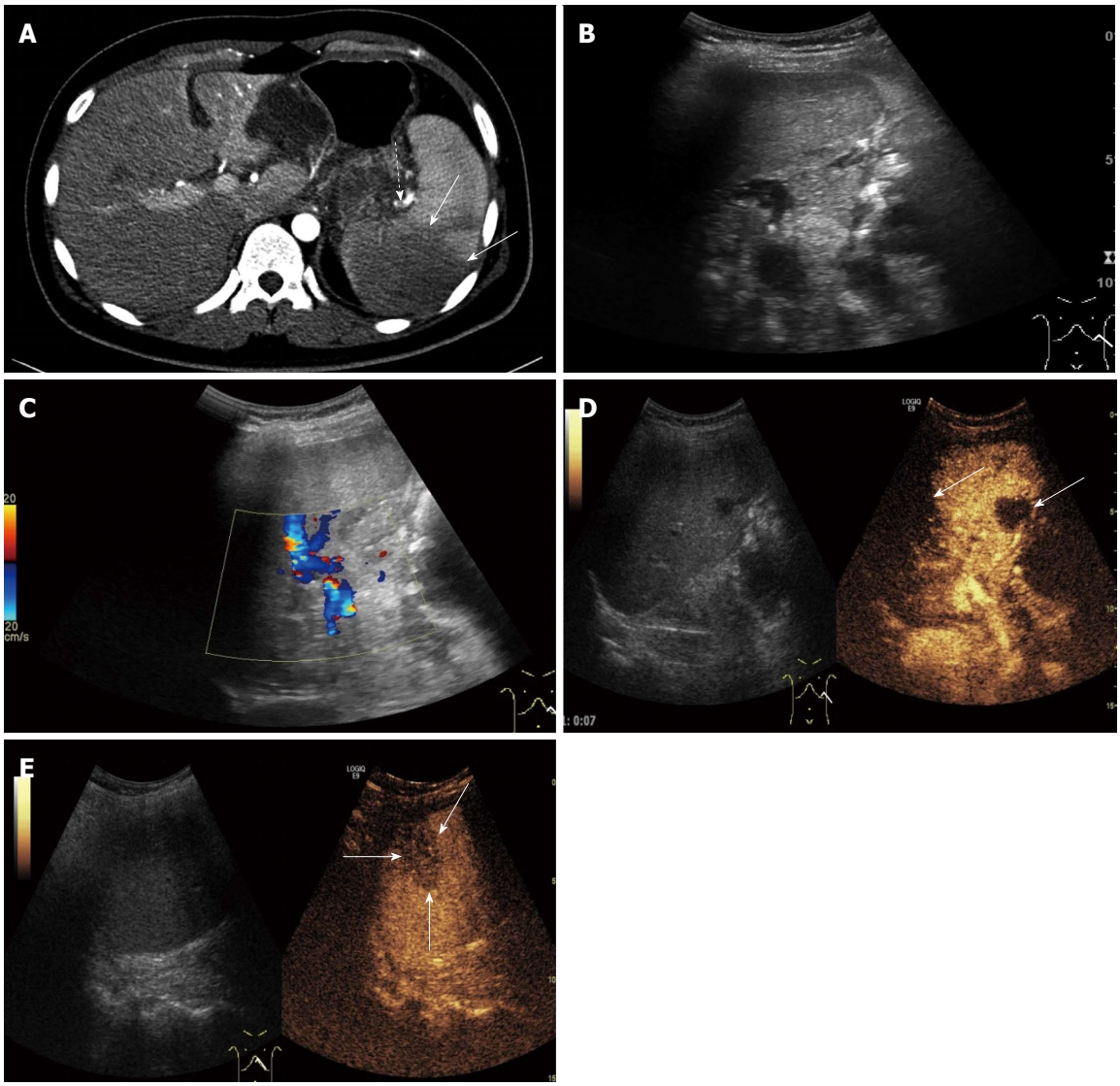


Figure 1 A 22-year-old man with splenic artery embolism. A: Enhanced-contrast computed tomography showing splenic artery embolism (dotted arrow), and the lesion of splenic infarction (solid arrow); B, C: Gray scale ultrasonography and color Doppler ultrasonography of the spleen showed no obvious lesion of splenic infarction; D: Some regions with no enhancement appeared in the splenic arterial phase (solid arrow) on contrast enhanced ultrasound (CEUS); E: A wedge-shaped splenic infarction with no enhancement was displayed in the splenic venous phase (solid arrow) on CEUS.

viously higher than that of pancreatic CEUS (0%, 0/12).

By splenic CEUS, 4 cases were diagnosed as splenic infarction, including 1 case complicated with sub-capsular hemorrhage, and 1 case of focal dysperfusion of the splenic parenchyma (Table 3). The CEUS imaging of splenic infarction showed that in some regions, no enhancement appeared after contrast agent injection in the splenic artery phase and venous phase, and hence infarcted splenic parenchyma was displayed as a hypoechoic area. The shape of splenic infarction varied from wedge-shaped (3 cases) to round or irregular (1 case).

Sub-capsular hemorrhage is a complication of splenic artery embolism. CEUS imaging of sub-capsular hemorrhage showed an anechoic region under the splenic subcapsule, which could differentiate better than pancreatic ultrasound, and there appeared an anechoic region and no enhancement after contrast agent injection in the splenic arterial phase and venous phase. There was one case diagnosed by CEUS as splenic infarction in the splenic CEUS group.

The imaging of focal dysperfusion of the splenic parenchyma revealed a region where the degree of enhancement inside the lesion area was lower than that of normal surrounding splenic parenchyma in the arterial and venous phases (especially the venous phase). The region of focal dysperfusion of the splenic parenchyma was displayed as a slightly hypoechoic area on CEUS, which did not appear on pancreatic ultrasound. Hence, a case of SAC was diagnosed by splenic CEUS, and there was no evidence of splenic rupture during his stay in hospital.

Five cases of splenic artery aneurysms were all missdiagnosed by pancreatic CEUS and splenic CEUS in AP. Because the whole splenic artery is difficult to display by ultrasound, especially the region which is close to the hiluslienis, gray scale ultrasound or CEUS may be necessary to identify the size or the location of splenic artery

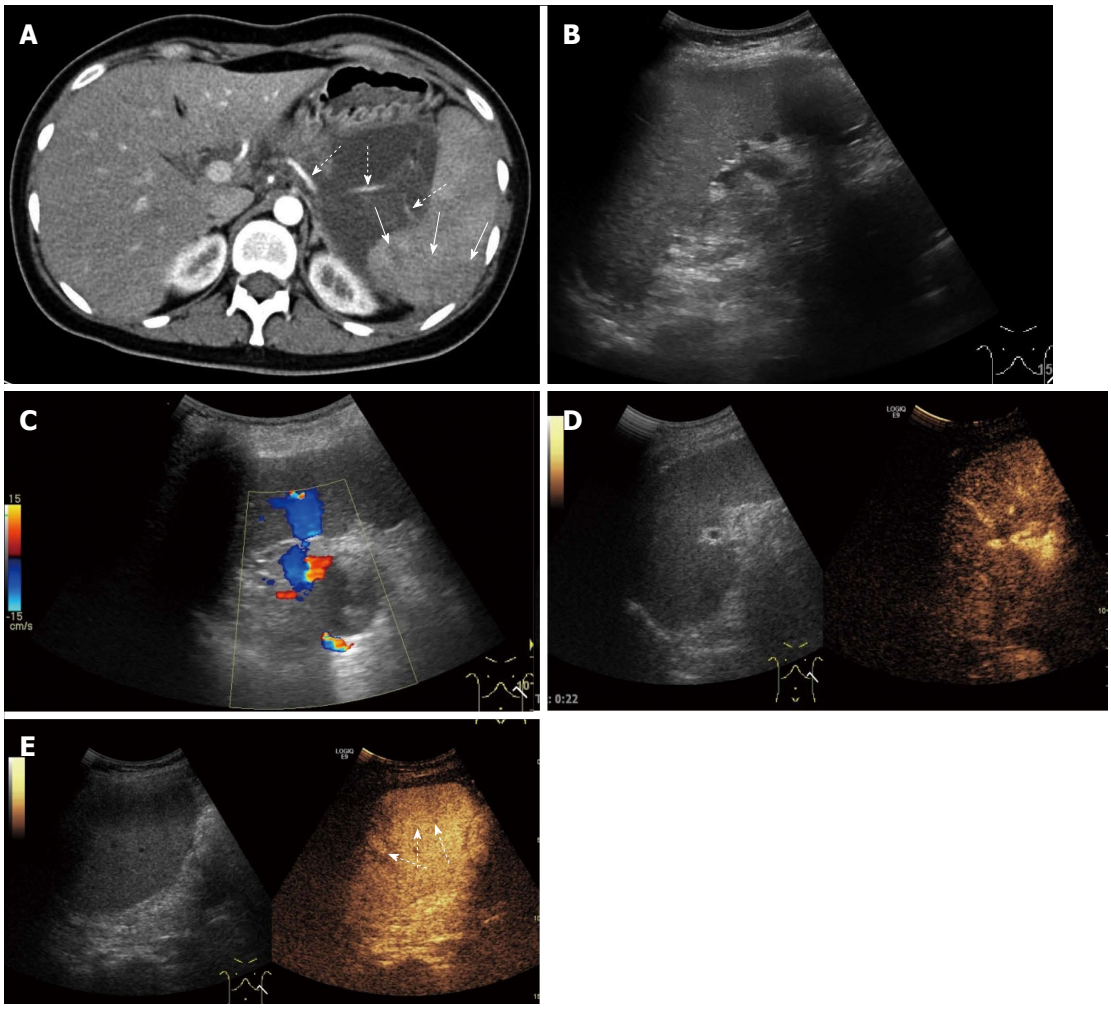


Figure 2 A 26-year-old woman with splenic arterial stenosis. A: Enhanced-contrast computed tomography showing splenic arterial stenosis (dotted arrow) and a focal splenic parenchyma region showed low enhancement (solid arrow); B, C: Gray scale ultrasonography and color Doppler ultrasonography showed no obvious lesions in the spleen; D: No obvious lesion was displayed in the splenic arterial phase on contrast enhanced ultrasound (CEUS); E: Some regions of focal dysperfusion of the splenic parenchyma showed low enhancement in the splenic venous phase (dotted arrow).

aneurysms.

DISCUSSION

AP is a common abdomen emergency and SACs in AP are rarely seen with a low incidence[4]. Due to the low sensitivity of ultrasound in diagnosing SACs, the missed diagnosis of the complications is often ignored[5], even by CEUS[6]. CECT was regarded as the gold standard to evaluate AP, especially the degree of severity[7,8]. CEUS changes the conventional viewpoint that ultrasound can only examine AP roughly[1,9], such as the pancreas size, shape, and peri-pancreatic fluid collection[10]. With the development of contrast agents, corresponding contrast-enhanced software and ultrasound equipment, CEUS is being utilized in more and more diseases, including pancreatic diseases, especially examination of SAP because it can display pancreatic parenchymal necrosis and the degree of necrosis, and the extra-pancreatic acute fluid collection. Previous studies concluded that ultrasound severity index (USSI) has a strong correlation with CT severity index (CTSI) and has been regarded as a substitute for CECT[6,9]. Only a few cases have been reported worldwide about CEUS for SACs compared with CECT for splenic venous complications[11-13]. The reason may be that the splenic artery is located between the tail of the pancreas and the hilum of the spleen with the body of the stomach in front. Gray scale sonography is influenced greatly by meteorism in the gastrointestinal tract, and diagnosis is sometimes difficult[14]. Because the quality of CEUS depends on the gray scale sonography, its quality is consequently affected, and the diagnostic accuracy for SACs is often ignored compared with CT or MRI[15].

Although the whole splenic artery is difficult to be displayed directly by ultrasound, we convert the observational objective to evaluate the lesions of the splenic artery indirectly. The diagnostic criteria for SACs were defined by observing the pattern of images obtained from the splenic CEUS. Blood supply to the spleen is solely from the splenic artery. Any lesions or changes in the splenic artery may result in splenic parenchymal infarction. In AP, splenic parenchymal infarction occurs due to

Table 2 Splenic artery complications examined by three methods in acute pancreatitis

	Splenic artery embolism	Splenic artery aneurysms	Splenic artery stenosis
CECT	6	5	1
Pancreatic CEUS	0	0	0
Splenic CEUS	4	0	1

CECT: Contrast-enhanced computed tomography; CEUS: Contrast enhanced ultrasound.

Table 3 Splenic artery complications by splenic contrast enhanced ultrasound in acute pancreatitis

Imaging of splenic CEUS	No.
Splenic infarction	4
Subcapsular hemorrhage	1
Splenic parenchymal dysperfusion	1
Splenic rupture	0
Splenic artery aneurysms	0

CEUS: Contrast enhanced ultrasound.

SACs. SACs are infrequent complications with pancreatitis, which mainly occur in chronic pancreatitis and SAP[16]. Mortelé *et al*[17] found that the incidence of splenic infarction in AP was about 7%, and there was a strong relationship between this complication and the severity of pancreatitis. In our study, 6 cases were diagnosed as splenic infarction by CECT. The incidence of splenic infarction in AP was 5.1% (6/118) and all cases with the complications were SAP. The findings in our study are consistent with the results reported by Mortelé *et al*.

SACs include splenic artery embolism, splenic artery aneurysms and splenic arterial stenosis[18]. SACs can lead to splenic infarction, sub-capsular hemorrhage, splenic rupture, or splenic aneurysm rupture. Color Doppler ultrasound is less sensitive to display acute infarction because there is no difference between the echo area of infarcted and normal parenchyma[19]. Acute splenic arterial stenosis is the main presentation of SAP. With the development of CEUS, splenic trauma or aortic aneurysm rupture can be diagnosed in clinical practice[20-22]. The microcirculation of the splenic parenchyma can be displayed by CEUS and it is possible to observe the ischemic alteration of the splenic parenchyma. These characteristics guarantee the diagnosis of acute infarction as early as possible and SAPs indirectly. The sensitivity of splenic CEUS for detecting SACs has increased obviously, and the accuracy rate was 41.7% (5/12, including 4 cases of splenic artery embolism and 1 case of splenic arterial stenosis) in diagnosing SACs compared with CECT.

There were no SACs diagnosed by pancreatic CEUS, indicating that pancreatic CEUS is a low sensitive method to diagnose SACs in AP. This finding also demonstrates that splenic CEUS is an indispensable technique in diagnosis of SACs in AP. It is of great clinical significance to optimize methods of diagnosis for AP complications, such as CEUS. Splenic CEUS is a felicitous supplement to overcome the disadvantage of pancreatic CEUS for SAPs in AP.

However, this study of diagnosis for splenic artery aneurysms is not satisfactory. In this study, all cases of splenic artery aneurysms were miss- diagnosed. We finally analyzed the reason for missed diagnosis of splenic artery aneurysms. The longest diameter of splenic artery aneurysms was less than 2 cm and the location was far from the hilum of the spleen so that sonologists could not observe the lesions, even by CEUS. And there were 2 cases of splenic infarction which were miss-diagnosed by splenic CEUS. We retrospectively analyzed the reasons why splenic CEUS missed the diagnosis. The reason was that the splenic infarction in 2 cases was located close to the diaphragm and at the sub-capsular area of the spleen, where ultrasound imaging would be obstructed by the ribs. Sonologists did not notice the missed area, which was just located in the shadow of the imaging. By controlling the quality of ultrasound imaging and avoiding the influence of the ribs, the incidence of missed diagnosis would be decreased.

The ultrasound contrast agent (suspension made of 5 mL normal saline mixed with SonoVue dry powder) was administered by intravenous injection at 1.5-2.0 mL for each pancreatic CEUS examination. Pancreatic CEUS examination generally did not run out of the suspension. Usually the remaining suspension of about 1.0-2.0 mL was sufficient for splenic CEUS examination. Therefore, it is economic to perform the splenic CEUS after pancreatic CEUS examination. Splenic CEUS examination could diagnose not only splenic artery lesions but also any other pathological changes, such as pancreatic pseudocysts in the splenic parenchyma[23,24].

Although CECT is the gold standard in diagnosing AP, ultrasound has an important position in pancreatitis examination[25]. When the patient's condition is not fit for CECT, such as iodine allergy, unavailability for bedside examination, and cases of renal insufficiency, CEUS is an effective substitute for CECT examination[26,27].

In conclusion, splenic CEUS diagnosis of SACs in AP was better than pancreatic CEUS because it had a higher diagnostic accuracy than the latter. In splenic CEUS examination, the contrast agent can be utilized more effectively and economically. Splenic CEUS can obviously increase the diagnostic accuracy for SACs in AP, which is simple, convenient, efficient, safe, and partly solves the disadvantages of other modalities in diagnosis of SACs in AP. Splenic CEUS is a supplementary method for pancreatic CEUS when AP patients need pancreatic CEUS examination, which can decrease the missed diagnosis of SACs.

ACKNOWLEDGMENTS

The authors are indebted to Professor Qin Xia, Zhong-Wen Huang, and Wen-Fu Tang (Department of Integrated Chinese and Western Medicine of West China Hospital) for providing the cases in this study. We also

thank Lin Lan and Xiao-Ying Feng for assistance with the injection of contrast agents.

COMMENTS

Background

Splenic artery complications (SACs) have a low morbidity in acute pancreatitis (AP). Although SACs are rare complications in AP and most patients present few clinical symptoms, they may lead to a severe outcome. Rupture of splenic artery aneurysms is frequent and sometimes occurs as the first symptom, and sometimes it is fatal. However, the sensitivity of ultrasound is low, even for pancreatic contrast-enhanced ultrasound (CEUS). The authors introduced an indirect method used for splenic CEUS to show the SACs in AP.

Research frontiers

CEUS has been used to reflect the necrosis range of the pancreas in AP. The enhancement patterns of AP have been described previously. However, the diagnostic value of splenic CEUS for SACs in AP has not been investigated or reported in the English-language literature.

Innovations and breakthroughs

The diagnostic value of splenic CEUS for SACs in AP was discussed in this study. When the splenic parenchyma shows a lower enhanced area than the surrounding parenchyma, or an unenhanced area of the infarct lesion or an unenhanced area of the fluid collection under the splenic subcapsule, a diagnosis of SACs in AP should be suspected.

Applications

Splenic CEUS is a convenient and useful method for the detection and discrimination of SACs in AP. SACs could be better managed if ultrasound technicians and physicians are familiar with their features on CEUS.

Terminology

Contrast enhanced ultrasonography (CEUS) is the application of an ultrasound contrast agent to traditional color Doppler sonography. Microbubble contrast agents produce a unique sonogram with increased contrast due to the high echogenicity difference. CEUS can be used to image blood perfusion in organs and tissues.

Peer review

This is a well written and interesting paper.

REFERENCES

1 **Ripollés T**, Martínez MJ, López E, Castelló I, Delgado F. Contrast-enhanced ultrasound in the staging of acute pancreatitis. *Eur Radiol* 2010; **20**: 2518-2523 [PMID: 20532782]

2 **Carr SC**, Mahvi DM, Hoch JR, Archer CW, Turnipseed WD. Visceral artery aneurysm rupture. *J Vasc Surg* 2001; **33**: 806-811 [PMID: 11296336]

3 **Rickes S**, Rauh P, Uhle C, Ensberg D, Mönkemüller K, Malfertheiner P. Contrast-enhanced sonography in pancreatic diseases. *Eur J Radiol* 2007; **64**: 183-188 [PMID: 17869470 DOI: 10.1016/j.ejrad.2007.06.035]

4 **Horváth K**, Györi-Molnár L, Bíró E, György E. [Ultrasonic diagnosis of splenic complications of chronic pancreatitis]. *Orv Hetil* 1986; **127**: 2311-2312 [PMID: 3534684]

5 **Fishman EK**, Soyer P, Bliss DF, Bluemke DA, Devine N. Splenic involvement in pancreatitis: spectrum of CT findings. *AJR Am J Roentgenol* 1995; **164**: 631-635 [PMID: 7863884 DOI: 10.2214/ajr.164.3.7863884D]

6 **Lu Q**, Zhong Y, Wen XR, Huang ZW, Fan YT, Xia Q, Luo Y. Can contrast-enhanced ultrasound evaluate the severity of acute pancreatitis? *Dig Dis Sci* 2011; **56**: 1578-1584 [PMID: 21113802 DOI: 10.1007/s10620-010-1460-6]

7 **Whitcomb DC**. Clinical practice. Acute pancreatitis. *N Engl J Med* 2006; **354**: 2142-2150 [PMID: 16707751]

8 **Balthazar EJ**. Acute pancreatitis: assessment of severity with clinical and CT evaluation. *Radiology* 2002; **223**: 603-613 [PMID: 12034923 DOI: 10.1148/radiol.2233010680]

9 **Rickes S**, Uhle C, Kahl S, Kolfenbach S, Monkemuller K, Effenberger O, Malfertheiner P. Echo enhanced ultrasound: a new valid initial imaging approach for severe acute pancreatitis. *Gut* 2006; **55**: 74-78 [PMID: 16033880 DOI: 10.1136/gut.2005.070276]

10 **Gandolfi L**, Torresan F, Solmi L, Puccetti A. The role of ultrasound in biliary and pancreatic diseases. *Eur J Ultrasound* 2003; **16**: 141-159 [PMID: 12573783 DOI: 10.1016/S0929-8266(02)00068-X]

11 **Malka D**, Hammel P, Lévy P, Sauvanet A, Ruszniewski P, Belghiti J, Bernades P. Splenic complications in chronic pancreatitis: prevalence and risk factors in a medical-surgical series of 500 patients. *Br J Surg* 1998; **85**: 1645-1649 [PMID: 9876067 DOI: 10.1046/j.1365-2168.1998.00952.x]

12 **Miller LA**, Mirvis SE, Shanmuganathan K, Ohson AS. CT diagnosis of splenic infarction in blunt trauma: imaging features, clinical significance and complications. *Clin Radiol* 2004; **59**: 342-348 [PMID: 15041453 DOI: 10.1016/j.crad.2003.09.005]

13 **Wang L**, Liu GJ, Chen YX, Dong HP, Wang LX. Sinistral portal hypertension: clinical features and surgical treatment of chronic splenic vein occlusion. *Med Princ Pract* 2012; **21**: 20-23 [PMID: 22024761 DOI: 10.1159/000329888]

14 **Rickes S**, Mönkemüller K, Malfertheiner P. Acute severe pancreatitis: contrast-enhanced sonography. *Abdom Imaging* 2007; **32**: 362-364 [PMID: 17514345 DOI: 10.1007/s00261-007-9250-0]

15 **Dietrich CF**. Comments and illustrations regarding the guidelines and good clinical practice recommendations for contrast-enhanced ultrasound (CEUS)--update 2008. *Ultraschall Med* 2008; **29** Suppl 4: S188-S202 [PMID: 18833497 DOI: 10.1055/s-2008-1027799]

16 **Patil PV**, Khalil A, Thaha MA. Splenic parenchymal complications in pancreatitis. *JOP* 2011; **12**: 287-291 [PMID: 21546711]

17 **Mortelé KJ**, Mergo PJ, Taylor HM, Ernst MD, Ros PR. Splenic and perisplenic involvement in acute pancreatitis: determination of prevalence and morphologic helical CT features. *J Comput Assist Tomogr* 2001; **25**: 50-54 [PMID: 11176293]

18 **Miller FH**, Keppke AL, Dalal K, Ly JN, Kamler VA, Sica GT. MRI of pancreatitis and its complications: part 1, acute pancreatitis. *AJR Am J Roentgenol* 2004; **183**: 1637-1644 [PMID: 15547203 DOI: 10.2214/ajr.183.6.01831637]

19 **Ray S**, Mridha AR, Ahammed M. Diffuse splenic infarction in a case of severe acute pancreatitis. *Am J Surg* 2011; **201**: e23-e25 [PMID: 21367360 DOI: 10.1016/j.amjsurg.2010.03.020]

20 **Tang J**, Zhang H, Lv F, Li W, Luo Y, Wang Y, Li J. Percutaneous injection therapy for blunt splenic trauma guided by contrast-enhanced ultrasonography. *J Ultrasound Med* 2008; **27**: 925-932; quiz 933 [PMID: 18499852]

21 **Wang D**, Lv F, Luo Y, An L, Li J, Xie X, Tian J, Zhao W, Tang J. Comparison of transcutaneous contrast-enhanced ultrasound-guided injected hemostatic agents with traditional surgery treatment for liver, spleen and kidney trauma: a retrospective study. *Hepatogastroenterology* 2012; **59**: 2021-2026 [PMID: 22591663 DOI: 10.5754/hge12255]

22 **Catalano O**, Lobianco R, Cusati B, Siani A. Contrast-enhanced sonography for diagnosis of ruptured abdominal aortic aneurysm. *AJR Am J Roentgenol* 2005; **184**: 423-427 [PMID: 15671357 DOI: 10.2214/ajr.184.2.01840423]

23 **Heider R**, Behrns KE. Pancreatic pseudocysts complicated by splenic parenchymal involvement: results of operative and percutaneous management. *Pancreas* 2001; **23**: 20-25 [PMID: 11451143 DOI: 10.1097/00006676-200107000-00003]

24 **Siablis D**, Papathanassiou ZG, Karnabatidis D, Christeas N, Katsanos K, Vagianos C. Splenic arteriovenous fistula and sudden onset of portal hypertension as complications of a ruptured splenic artery aneurysm: Successful treatment with transcatheter arterial embolization. A case study and review of the literature. *World J Gastroenterol* 2006; **12**: 4264-4266 [PMID: 16830391]

25 **Luo Y**, Yuan CX, Peng YL, Wei PL, Zhang ZD, Jiang JM,

Dai L, Hu YK. Can ultrasound predict the severity of acute pancreatitis early by observing acute fluid collection? *World J Gastroenterol* 2001; **7**: 293-295 [PMID: 11819778]

26 **Arvanitakis M**, Delhaye M, De Maertelaere V, Bali M, Winant C, Coppens E, Jeanmart J, Zalcman M, Van Gansbeke D, Devière J, Matos C. Computed tomography and magnetic resonance imaging in the assessment of acute pancreatitis. *Gastroenterology* 2004; **126**: 715-723 [PMID: 14988825 DOI: 10.1053/j.gastro.2003.12.006]

27 **Manfredi R**, Brizi MG, Canadè A, Vecchioli A, Marano P. Imaging of acute pancreatitis. *Rays* 2001; **26**: 135-142 [PMID: 11925784]

P- Reviewer: Poma EM **S- Editor**: Wen LL
L- Editor: Wang TQ **E- Editor**: Ma S

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.1095

World J Gastroenterol 2014 January 28; 20(4): 1095-1106
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

META-ANALYSIS

Combination of chemotherapy and immunotherapy for colon cancer in China: A meta-analysis

Zheng-Xu Wang, Jun-Xia Cao, Zhi-Ping Liu, Yu-Xin Cui, Chun-Yun Li, Duo Li, Xiao-Yan Zhang, Jin-Long Liu, Jun-Li Li

Zheng-Xu Wang, Jun-Xia Cao, Chun-Yun Li, Duo Li, Xiao-Yan Zhang, Jin-Long Liu, Jun-Li Li, Biotherapy Center, the General Hospital of Beijing Military Command, Beijing 100700, China
Zhi-Ping Liu, Department of Immunology, St Jude Children's Research Hospital, Memphis, TN 38105, United States
Yu-Xin Cui, School of Cellular and Molecular Medicine, University of Bristol, University Walk, Bristol BS8 1TD, United Kingdom
Author contributions: Wang ZX designed the research; Cao JX and Li CY performed the research; Liu ZP and Cui YX contributed new reagents or analytic tools; Cao JX, Li CY, Li D, Zhang XY, Liu JL and Li JL analyzed data; Wang ZX, Cao JX, Liu ZP and Cui YX wrote the paper.
Supported by National Natural Science Foundation of China, No. 31171427 and No. 30971651 to Wang ZX; National Natural Science Foundation of China, No. 30700974 to Cao JX; and Postdoctoral Foundation of China, No. 20060400775 to Cao JX
Correspondence to: Zheng-Xu Wang, MD, PhD, Associate Professor, Director of Biotherapy Center, the General Hospital of Beijing Military Command, No.5 of Nan Men Cang Rd, Dongcheng District, Beijing 100700, China. zhxwang18@hotmail.com
Telephone: +86-10-66721169 Fax: +86-10-64038276
Received: September 10, 2013 Revised: October 28, 2013
Accepted: November 12, 2013
Published online: January 28, 2014

Abstract

AIM: To investigate whether autologous dendritic cell (DC)-cytokine-induced killer (CIK) cell therapy is able to improve the therapeutic efficacy of chemotherapy in colon cancer.

METHODS: We conducted a systematic review of published papers from the sources of MEDLINE, the Cochrane Central Register of Controlled Trials, EMBASE, the Wanfang Database, the China Science and Technology Periodical Database and China Journal Net. Published data were extracted independently by two authors using predefined database templates. The quality of the data from individual papers was also assessed. The effects of chemotherapy were compared with those of chemotherapy in combination with DC-CIK immunotherapy. The pooled analysis was performed using the data from random or fixed-effect models.

RESULTS: Seven trials matched our inclusion criteria (n = 533). The overall analysis showed significant survival benefit [one-year overall survival (OS), $P < 0.0001$; two-year OS, $P = 0.009$; three-year OS, $P = 0.002$] in favor of DC-CIK immunotherapy combined with chemotherapy. Disease-free survival (DFS) rate was improved after the combination of DC-CIK immunotherapy and chemotherapy (one-year DFS, $P < 0.0001$; two-year DFS, $P = 0.002$; three-year DFS, $P = 0.02$). An improved overall response rate ($P = 0.009$) was also observed in patients who received DC-CIK therapy. Furthermore, the analysis of T-lymphocyte subsets in peripheral blood indicated that the number of $CD4^+$ T cells significantly increased in the DC-CIK plus chemotherapy group ($P < 0.05$).

CONCLUSION: The combination of DC-CIK immunotherapy and chemotherapy was superior in prolonging the survival time and enhancing immunological responses.

Key words: Dendritic cells; Cytokine-induced killer cells; Meta-analysis; Colon cancer; Immunotherapy

Core tip: A growing body of knowledge on tumor immunosurveillance and loss thereof has contributed to the refinement of anti-tumor immunotherapy. The aim of our meta-analysis was to determine whether an association exists between dendritic cell (DC)-cytokine-induced killer (CIK) cell therapy combined with chemotherapy and chemotherapy alone. Our analysis demonstrates that DC-CIK therapy improved 1, 2 and 3-year overall

survival, 1, 2 and 3-year disease-free survival, overall response rate and immune indices in colon cancer. In all, the combination of DC-CIK immunotherapy and chemotherapy was superior in prolonging the survival time and enhancing immunological responses, suggesting the possible application of this promising adjuvant immunotherapy in colon cancer.

Wang ZX, Cao JX, Liu ZP, Cui YX, Li CY, Li D, Zhang XY, Liu JL, Li JL. Combination of chemotherapy and immunotherapy for colon cancer in China: A meta-analysis. *World J Gastroenterol* 2014; 20(4): 1095-1106 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/1095.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.1095

INTRODUCTION

Colorectal cancer is the third most commonly diagnosed cancer in humans. As dietary habits have changed in recent years, the number of cases of colon cancer have been increasing faster in the Eastern world[1,2]. Although surgical resection is the first choice worldwide, colorectal cancer can also be treated effectively with chemotherapy, radiation therapy, or both to improve patient survival. However, 25% of patients that present with metastatic disease have a five-year survival of only 10%. A variety of therapeutic strategies for metastatic colon cancer have been evaluated over the last decade, however, most patients in advanced stages of the disease have little hope of longer survival. Therefore, an effective approach for the treatment of colorectal cancer patients with metastasis and post-operative cancer recurrence is critical. In recent years there has been great interest in cancer immunotherapy, which has the potential to control metastatic disease, prolong time to recurrence, and ultimately serve as a preventive measure.

Adoptive immunotherapy holds great promise in the scenario of potential new approaches for the treatment of solid tumors that are refractory to conventional therapies. Recently, the effectiveness of immunotherapy in the treatment of numerous forms of cancers has been studied. For instance, it was reported that adoptive cell transfer of *ex vivo*-activated autologous tumor-reactive Tc1 or Tc17 T cells can mediate effective anti-tumor immunity and tumor regression in melanoma[3]. Cytokine-induced killer (CIK) cell therapy is also able to induce complete clinical responses in renal cell carcinoma (RCC) patients, and was demonstrated to be safe and effective in these patients[4]. A systematic review and meta-analysis of randomized controlled trials in multiple myeloma (MM) indicate that autologous-allogeneic (auto-allo) hematopoietic cell transplantation (HCT) can induce higher complete remission rates. There is no improvement in overall survival (OS) with auto-allo HCT, however, this approach can achieve higher non-relapse mortality rates in patients with newly diagnosed MM[5]. Another study suggested that erythropoietin-producing hepatocellular carcinoma A2 (EphA2)-specific T-cell immunotherapy may be a promising approach for the treatment of EphA2-positive glioblastoma[6]. Furthermore, αβ and γδ T cell-based immunotherapy has been found to improve treatment efficacy in osteosarcoma, especially at the recurrent and metastatic stages[7]. A multi-center historical cohort study indicated that the effectiveness of immunotherapy in advanced lung cancer is limited, but may extend life span under certain conditions. Moreover, immunotherapy can maintain good quality of life in patients until near the time of death[8]. The bedside medication administration of Sipuleucel-T (Provenge, Dendreon) was the first-in class therapeutic autologous vaccine to be approved for the treatment of men with asymptomatic or minimally symptomatic castrate-resistant metastatic prostate cancer in the spring of 2010[9]. This product represents the culmination of basic immunological and prostate cancer investigations for decades, and 13 years of clinical trials[10]. Another immunotherapeutic drug, the cytotoxic T-lymphocyte antigen 4 antibody, Ipilimumab, has been demonstrated to improve OS in patients with metastatic melanoma in two phase Ⅲ trials[11,12] and was approved in March of 2011. These important drug development milestones provide solid evidence that targeting of the immune system can lead to clinically relevant immune responses, thus extending the life span of cancer patients.

Challenging issues for all adoptive immunotherapy strategies include the obtainment of sufficient numbers of immune effectors, recognition of tumor targets and possible restriction to specific human leukocyte antigens-haplotypes. Such hurdles have also been encountered in immunotherapy trials for colon cancer. CIK cells are *ex vivo*-expanded T lymphocytes that share phenotypic and functional properties with both natural killer and T cells. It has been reported that secretory glycoprotein 90K-specific cytotoxic T lymphocytes (CTLs) generated by 90K-pulsed dendritic cells (DCs) are useful effector cells for immunotherapy in colon cancer[13]. Mucin 1 (CD227)-specific CTLs have also been shown to cause complete rejection of tumor cells, when in the therapeutic regimen, and tumor burden was significantly reduced[14]. CTLs specific for the tumor-associated antigen, CEP55, can efficiently recognize colon cancer stem-like cells or tumor-initiating cells which highly express the stem cell markers, SRY-box 2, POU class 5 homeobox 1, leucine-rich repeat-containing G protein-coupled receptor 5 and aldehyde dehydrogenase 1 family member A1 *in vitro* and *in vivo*[15]. DCs are rare leucocytes that are uniquely potent in their ability to capture, process and present antigens to T cells. They selectively migrate through tissues to reach lymph nodes and spleen where initiation of the immune response takes place. DC-based vaccinations are an attractive candidate adjunct therapy to treat colon cancer patients. It has been demonstrated that signal transducers and activators of transcription 3-depleted DC vaccination induces effective systemic anti-tumor effects through high antigen (Ag) -specific T cell responses accompanied by systemic T helper 1 immune responses in a murine colon cancer model[16]. DCs

pulsed with carcinoembryonic antigen (CEA) peptide resulted in prolonged antigen-presentation and efficient T-cell activation, but not CEA mRNA for vaccination[17]. A phase Ⅰ-Ⅱ CEA-loaded DC vaccine trial in patients with colon cancer is ongoing (www.clinicaltrial.gov id: NCT 01219348). In the microenvironment of human colon adenocarcinoma, the supernatant mediates endothelial-like differentiation of induced DCs by extracellular signal-regulated protein kinases 1 and 2 signaling, which suggests that immunocytes are involved in the cancer microenvironment[18]. Based on findings in the tumor microenvironment and cancer stem cells in cancer therapy, immunotherapy is worth further investigation.

Over the past few years, advances in our understanding of the immune system, improved design of clinical trials, and improvement and compliance of manufacturing processes have provided opportunities to significantly improve efficacy and safety of immunotherapy. However, clinical studies on DC-CIK cells are still in their infancy and there is no clear consensus on how they may be best optimized. Therefore, we performed a systematic review and meta-analysis of clinical trials to assess the therapeutic efficacy of DC-CIK cells combined with chemotherapy in colon cancer.

MATERIALS AND METHODS

Search strategy and selection criteria

Trials were identified by electronic search in the PubMed database (1976 onward), Embase (1966 onward), the Cochrane Central Registry of Controlled Trials (no date restriction), the Wanfang Database (no date restriction), the China Science and Technology Periodical Database (no date restriction), China Journal Net (no date restriction), reference lists of published trials and relevant review articles. The search strategy included the following medical subject headings: "colon cancer", "cytokine-induced killer cells", "dendritic cells", "immunotherapy", "colon rectal cancer" and free text search. No language limits were applied. The initial search was performed in June 2012 and updates were conducted in June 2013. Furthermore, we contacted drug manufacturers, asked experts in the field, and performed manual searches in reference lists, conference proceedings of the American Society of Clinical Oncology Annual Meetings and the European Cancer Conference. We also searched the http://www.ClinicalTrials.gov website for information on prospective and ongoing trials. No language restriction was applied. We excluded abstracts that were never subsequently published as full papers and studies on animals and cell lines.

Data extraction and quality assessment

Data extraction was independently conducted by two reviewers (Cao JX and Li CY) using a standardized approach. Disagreement was adjudicated by a third reviewer (Li D) after referring back to the original publications. We collected information including authors' names, journal, year of publication, sample size per arm, regimen used, median or mean age of patients, sex, numbers of patients assessable for 1- and 3-year overall survival and numbers of patients assessable for 3-year disease-free survival and information pertaining to study design (whether the trial reported the mode of randomization, allocation concealment, description of withdrawals per arm, and blinding) for the trials included in the study.

Definition of outcome measures

OS was defined as the time from the initiation of treatment until death. The secondary objective was disease-free survival (DFS). Other endpoints were disease control rate [DCR = complete response (CR) + partial response (PR) + stable disease (SD)] and objective response rate (ORR = CR + PR), respectively. CR, PR, mixed response or SD were documented and extracted for analysis.

Statistical analysis

Statistical analysis was carried out by pair-wise comparison of the immunotherapy-containing arms of the identified trials with the respective non-immunotherapy arms. Treatment effects are reflected by odds ratios (OR) for OS, DFS, ORR and clinical benefit rate. To calculate the pooled OR, the number of OS, DFS, ORR and DCR in each arm were extracted from each study and combined using a method reported by Mantel and Haenszel. A pooled OR < 1 indicated lower recurrence or lower survival in the immunotherapy arm. To evaluate whether the results of the studies were homogeneous, we used Cochran's Q test. This statistical test is a χ^2 test with df equal to the number of studies minus one, and tests the null hypothesis that the difference between the study estimates of OR is due to chance. We also calculated the quantity, I^2, which describes the percentage of variation across studies due to heterogeneity rather than chance. I^2 values of 25%, 50%, and 75% were used as evidence of low, moderate, and high heterogeneity, respectively. SPSS 11.5 was also used to carry out the data analysis. The OR was calculated with a fixed-effect model when no statistically significant heterogeneity existed; otherwise, a random-effect model was employed. P-values < 0.05 were considered statistically significant. All reported P-values resulted from two-sided version tests of the respective tests.

RESULTS

Selection of the trials

The electronic search yielded 147 references. After title and abstract review, 122 publications were excluded for various reasons (17 were review articles, 21 used animal models, 28 were case reports, 36 were *in vitro* experiments, 20 were nursing studies). The full texts of 25 articles were selected as potentially relevant and retrieved for more detailed assessment. We excluded a total of 18 studies as they did not include detailed patient clinical data or therapy response, and the phase I clinical study in Germany with CIK cells for colorectal cancer patients, which did not include a control arm[19]. The selection procedure is

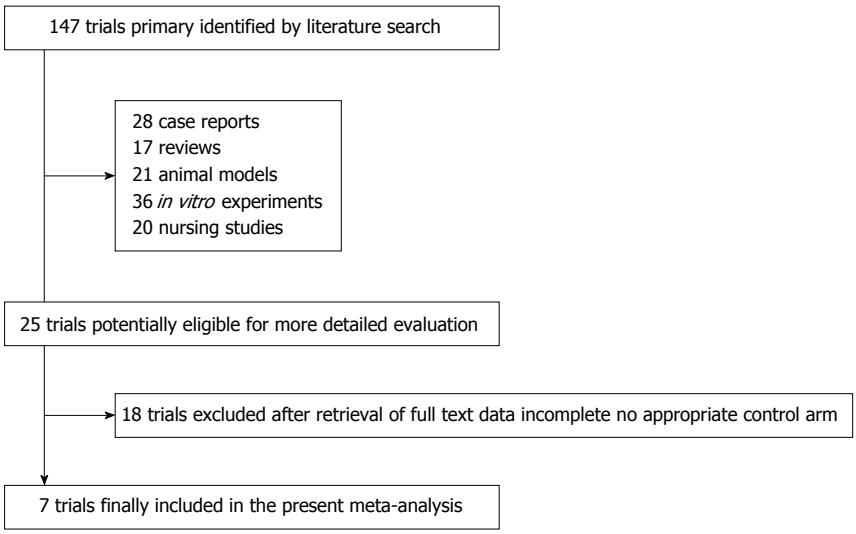


Figure 1 Flow diagram showing record identification, screening and study inclusion process.

shown in Figure 1. As a result, 7 articles reporting clinical trials of DC-CIK cell-based therapy combined with chemotherapy were selected for meta-analysis.

Characteristics of DC-CIK cell-based therapy

After the selection process, 7 eligible trials with a total of 533 patients were included in the present analysis. All of the trials were fully published. The seven selected papers contained four randomized studies, two retrospective analyses and one study considering treatment programs to construct the control. These studies are listed in Table 1 and other related clinical data are also listed.

Most of the patients in these studies had a good performance status with an expected duration of survival > 3 mo and the median age of the patients was 54.5 years. In all seven trials, DC-CIK or CIK cell therapy combined with chemotherapy was evaluated in patients with colon cancer. Interferon-γ, CD3 monoclonal antibody and interleukin (IL) -2 were used in the CIK cell culture systems in all the trials analyzed. In addition, granulocyte-macrophage colony stimulating factor (GM-CSF), IL-4, and tumor necrosis factor-α were used in the DC cell culture system. Both CIK and DC-CIK cell therapy were included in this analysis. In one of the trials, only CIK cell therapy was used for colon cancer treatment[20], while the other six trials utilized both DC and CIK cell therapy[21-26]. The number of CIK cells transfused into patients in these studies was more than 1.0×10^9/course.

Information on patients in the two groups (DC-CIK cell therapy combined with chemotherapy and chemotherapy alone) in these trials, such as gender, chemotherapy category (FOLFOX or XELOX and other chemicals) and CIK cell dose were analyzed by χ^2 test (data not shown). There was no statistically significant difference between the groups, with all *P*-values being > 0.05. The origins of the patient information from the articles in each group did not interfere with the results of the meta-analysis. However, the patient's age (including all the unknown patients) did impact on the efficacy of DC-CIK cell therapy by χ^2 test (data not shown). Furthermore, other clinical information from the trials such as tumor diameter and performance status were not analyzed due to insufficient data.

1-year overall survival

Information on 1-year survival was available in six trials[20-25]. These six trials included 491 patients (224 patients received immunotherapy combined with chemotherapy) in total. The 1-year overall survival rates were 93% (208/224) for colon cancer patients who received DC-CIK immunotherapy combined with chemotherapy. In comparison, 1-year overall survival rates were only 84% (224/267) in patients who did not receive DC-CIK immunotherapy. Each of the six trials showed longer survival for patients who received DC-CIK immunotherapy combined with chemotherapy. The estimated pooled OR for the six trials demonstrated a highly significant improvement in one-year survival for patients receiving DC-CIK immunotherapy combined with chemotherapy (OR 0.23; 95%CI: 0.11-0.48, $P < 0.0001$). The Cochran's *Q* test resulted in a *P* value of 0.53 and the corresponding quantity, I^2, was 0%, indicating that the degree of variability between the trials was consistent with what would be expected to occur by chance alone (Figure 2A).

2-year overall survival

Information on 2-year survival was available in four trials[21-25]. These studies included a collective total of 411 patients (184 patients received immunotherapy combined with chemotherapy) (Figure 2A). DC-CIK immunotherapy combined with chemotherapy resulted in 76% (140/184) of colon cancer patients achieving 2-year survival. In comparison, the 2-year overall survival for the control group was only 69% (157/227). The results of the pooled analysis showed that patients in the DC-CIK combined group had significantly improved two-year survival (OR = 0.42;

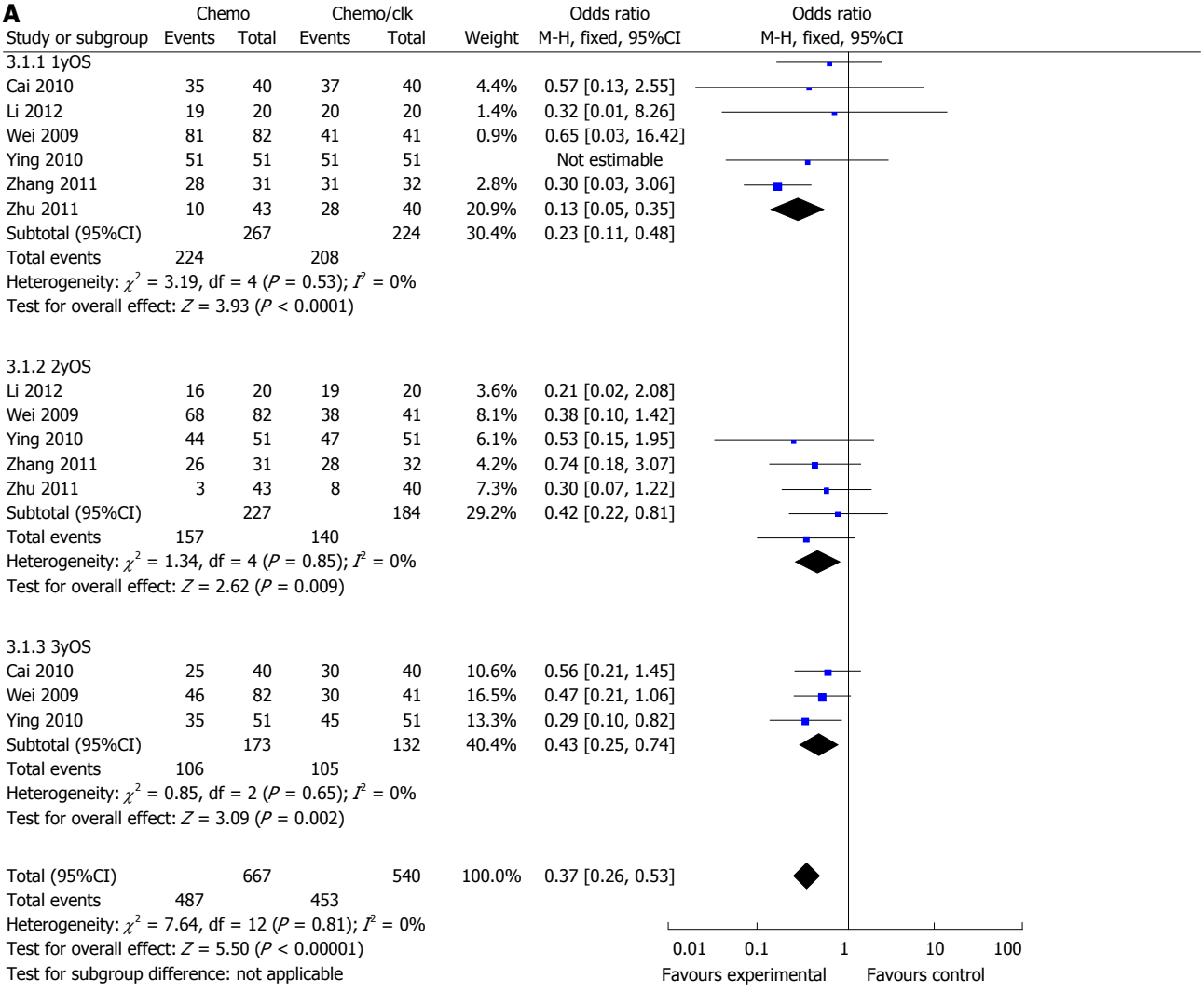


Figure 2 Overall survival. A: Comparison of 1-, 2- and 3-year overall survival (OS) between the chemo-dendritic cell-cytokine-induced killer cells and chemo-alone groups. The fixed-effects meta-analysis model (Mantel-Haenszel method) was used. Each trial is represented by a square, the center of which gives the odds ratio for that trial. The size of the square is proportional to the information in that trial. The ends of the horizontal bars denote a 95%CI. The black diamond gives the overall odds ratio for the combined results of all trials; B: Mean survival including two trials (Wei *et al*[22] and Ying *et al*[23]) with excel line graph. The dotted line represents the trend line of each group.

95%CI: 0.22-0.81, $P = 0.009$). There was no evidence of heterogeneity among individual studies ($P = 0.85$; $I^2 = 0\%$).

3-year overall survival

Information on 3-year survival was available in 3 trials[20,22,23], which included 305 patients (132 patients received immunotherapy combined with chemotherapy). DC-CIK immunotherapy combined with chemotherapy resulted in 80% (105/132) of colon cancer patients achieving 3-year overall survival, compared to 61% (106/173) in the che-

Table 1 Clinical information from the eligible trials

Ref.	Age (yr)	Tumor characteristic (TNM)	Regimens (per arm)	Patients (male)	Culture conditions of CIK cells	Culture conditions of DC cells	CIK regimens
Zhang *et al*[24]	UK	Ⅱ, Ⅲ, Ⅳ	Chemo Chemo-DC-CIK	31 (UK) 32 (UK) Randomized	IFNγ, CD3, IL1, IL-2	GM-CSF, IL-4, TNFα	> 1.0 × 10^9/course
Zhu *et al*[25]	58.3 (Mid) 59.2 (Mid)	Ⅱ, Ⅲ, Ⅳ	Chemo Chemo-DC-CIK	43 (27) 40 (24) Treatment program	IFNγ, CD3, IL-2	IFNγ, LPS	1.1-8.0 × 10^{10}/course
Ying *et al*[23]	UK	Ⅱ, Ⅲ	Chemo Chemo-DC-CIK	51 (25) 51 (31) Retrospective analysis	IFNγ, CD3, IL-1, IL-2	GM-CSF, IL-4, TNFα	⩾ 10^{10}/course
Yuan *et al*[26]	UK	Ⅲ, Ⅳ	Chemo Chemo-DC-CIK	21 (16) 21 (15) Randomized	IFNγ, CD3, IL-1, IL-2	GM-CSF, IL-4, TNFα, IFNγ	⩾ 10^{10}/course
Cai *et al*[20]	44.5 (Ave) 46.7 (Ave)	Ⅱ, Ⅲ	Chemo Chemo-CIK	40 (23) 40 (25) Randomized	IFNγ, CD3, IL-1, IL-2		UK
Wei *et al*[22]	55.5 (Mid) 54 (Mid)	Ⅰ, Ⅱ, Ⅲ	Chemo Chemo-DC-CIK	82 (41) 41 (18) Retrospective analysis	IFNγ, CD3, IL-1, IL-2	GM-CSF, IL-4, TNFα, IFNγ	⩾ 10^{10}/course
Li *et al*[21]	57.5 (Ave) 54.5 (Ave)	Ⅱ, Ⅲ	Chemo Chemo-DC-CIK	20 (15) 20 (13) Randomized	IFNγ, CD3, IL-2	GM-CSF, IL-4, IFNγ	UK

The table summarizes patient information regarding cases, age, details of the immunotherapy including dendritic cell (DC), cytokine-induced killer (CIK) cells or DC-CIK cells, and the culture conditions used for the cells. IFNγ: Interferon-gamma; IL: Interleukin; GM-CSF: Granulocyte-macrophage colony-stimulating factor; TNFα: Tumor necrosis factor-alpha; UK: Unknown; Ave: Average; Mid: Median.

motherapy only group. All three trials showed improved survival in the DC-CIK immunotherapy combined with chemotherapy patients. The estimated pooled OR for the three trials showed a highly significant improvement in three-year survival in patients receiving DC-CIK immunotherapy combined with chemotherapy (OR = 0.43; 95%CI: 0.25-0.74, $P = 0.002$) (Figure 2A). The Cochran's Q test resulted in a P value of 0.65 and the corresponding quantity, I^2, was 0%, indicating there was no evidence of heterogeneity among the individual studies.

Adequate information on the 1-year, 2-year and 3-year survival rate was only available in 2 trials (Wei *et al*[22] and Ying *et al*[23]), therefore, we simply summarized the data from these two trials to show the survival graph (Figure 2B). The data are shown on the figure and the dotted line represents the linear trend line ($r^2 = 0.9959$ for the immunotherapy group and $r^2 = 0.9854$ for the control group).

1-year disease-free survival

Information on 1-year DFS was available in two trials[22,23] and included 225 patients (92 patients received immunotherapy combined with chemotherapy) (Figure 3A). DC-CIK immunotherapy combined with chemotherapy led to 1-year DFS in 86% (79/92) of colon cancer patients. In contrast, 1-year DFS was only 63% (84/133) in patients who received only chemotherapy. Both trials showed longer DFS in DC-CIK immunotherapy combined with chemotherapy patients in comparison to chemotherapy only in the first year. The estimated pooled OR for the two trials showed a highly significant improvement in one-year DFS in patients receiving DC-CIK immunotherapy combined with chemotherapy (OR = 0.24; 95%CI: 0.120.49, $P < 0.0001$). The Cochran's Q test resulted in a P value of 0.47 and corresponding quantity, I^2, was 0%, indicating that the degree of variability between trials was consistent with what would be expected to occur by chance alone.

2-year disease-free survival

Information on 2-year DFS was available for two trials[22,23] and included 225 patients (92 patients received immunotherapy combined with chemotherapy). DC-CIK immunotherapy plus chemotherapy resulted in 60% (55/92) of colon cancer patients achieving 2-year DFS, compared to 40% (53/133) in the control group. The estimated pooled OR for the two trials showed a highly significant improvement in two-year DFS in patients receiving DC-CIK immunotherapy combined with chemotherapy (OR = 0.41; 95%CI: 0.23-0.71, $P = 0.002$). The Cochran's Q test resulted in a P value of 0.98 and corresponding quantity, I^2, was 0% (Figure 3A).

3-year disease-free survival

Information on 3-year DFS was available in two trials[22,23] and included 225 patients (92 patients received immunotherapy combined with chemotherapy). Immunotherapy plus chemotherapy resulted in 50% (46/92) of colon cancer patients achieving 3-year DFS. In contrast, chemotherapy alone resulted in 3-year DFS in only 36% (48/133) of control patients. The estimated pooled OR for the two trials showed a highly significant improvement in 3-year DFS in patients receiving DC-CIK immunotherapy combined with chemotherapy (OR = 0.50; 95%CI: 0.29-0.88, $P = 0.02$) (Figure 3A). The Cochran's Q test resulted in a P value of 0.99 and the correspond-

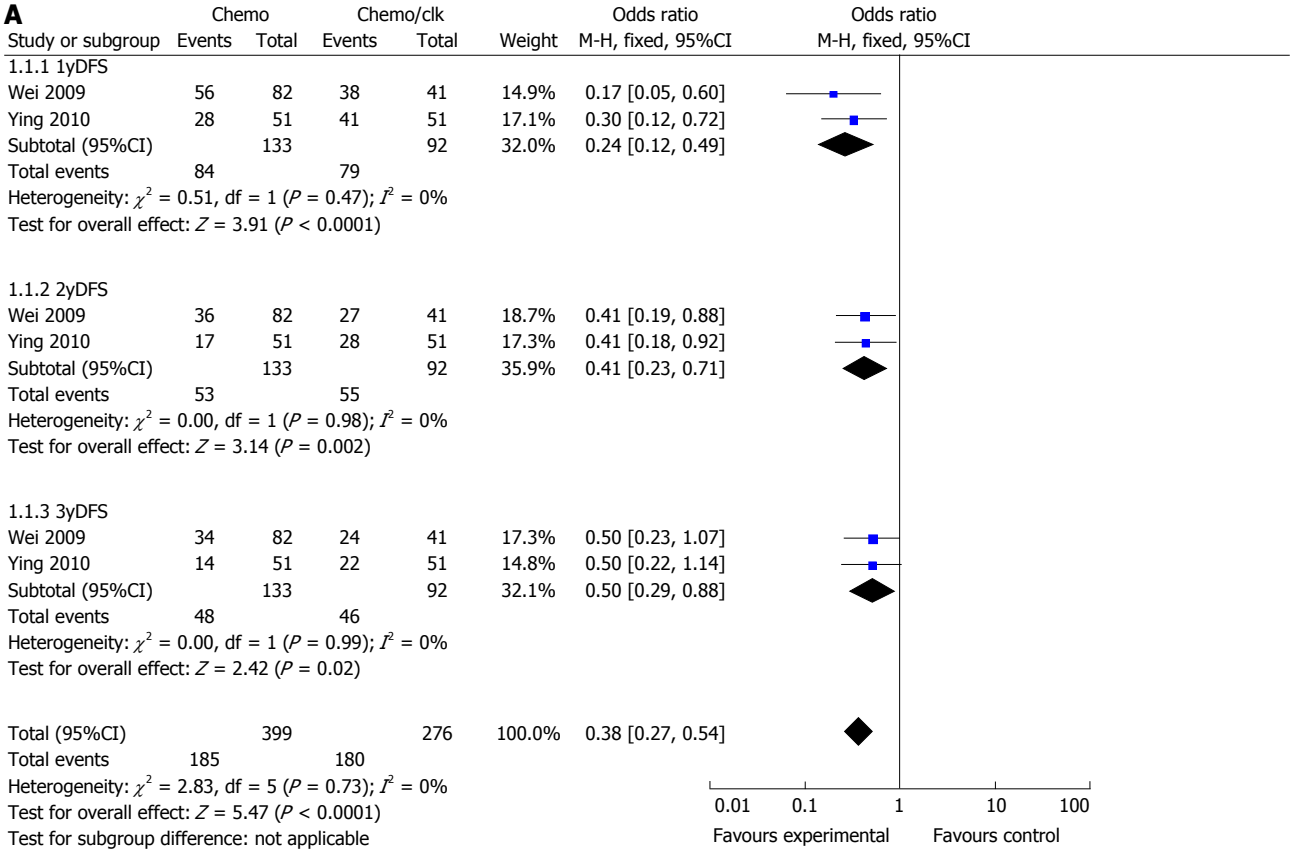


Figure 3 Disease-free survival. A: Forest plot for disease-free survival (DFS). The fixed effects model (Mantel-Haenszel method) was used in this analysis; B: Mean DFS including two trials (Wei *et al*[22] and Ying *et al*[23]) with excel line graph. The dotted line represents the trend line of each group.

ing quantity, I^2, was 0%, indicating that there was no evidence of heterogeneity among the individual studies.

Adequate information on 1-year, 2-year and 3-year DFS was only available in 2 trials (Wei *et al*[22] and Ying *et al*[23]), therefore, we simply summarized the data from these two trials to show the DFS graph (Figure 3B). The data are shown on the figure and the dashed line represents the linear trend line (r^2 = 0.9382 for the immunotherapy group and r^2 = 0.8583 for the control group).

Response rate

Analysis of the ORR also demonstrated favorable results for the DC-CIK therapy arm, with an OR of 0.35 (95%CI: 0.16-0.77, P = 0.009). However, the DCR for the chemotherapy combined with DC-CIK group did not significantly differ from the chemotherapy alone group (OR = 0.54; 95%CI: 0.21=1.43, P = 0.22) (Figure 4). The Cochran's Q test resulted in a P value of 0.80 and 0.68, respectively, while the corresponding quantity, I^2, was 0% for both groups, indicating that there was no evidence of heterogeneity among the individual studies.

Comparison of lymphocyte/monocyte subsets in the peripheral blood of cancer patients

Analysis showed that the proportion of $CD4^+$ cells was significantly increased in the DC-CIK group compared with corresponding baseline percentages before treatment, which was reflected by the pooled OR of -6.80 for

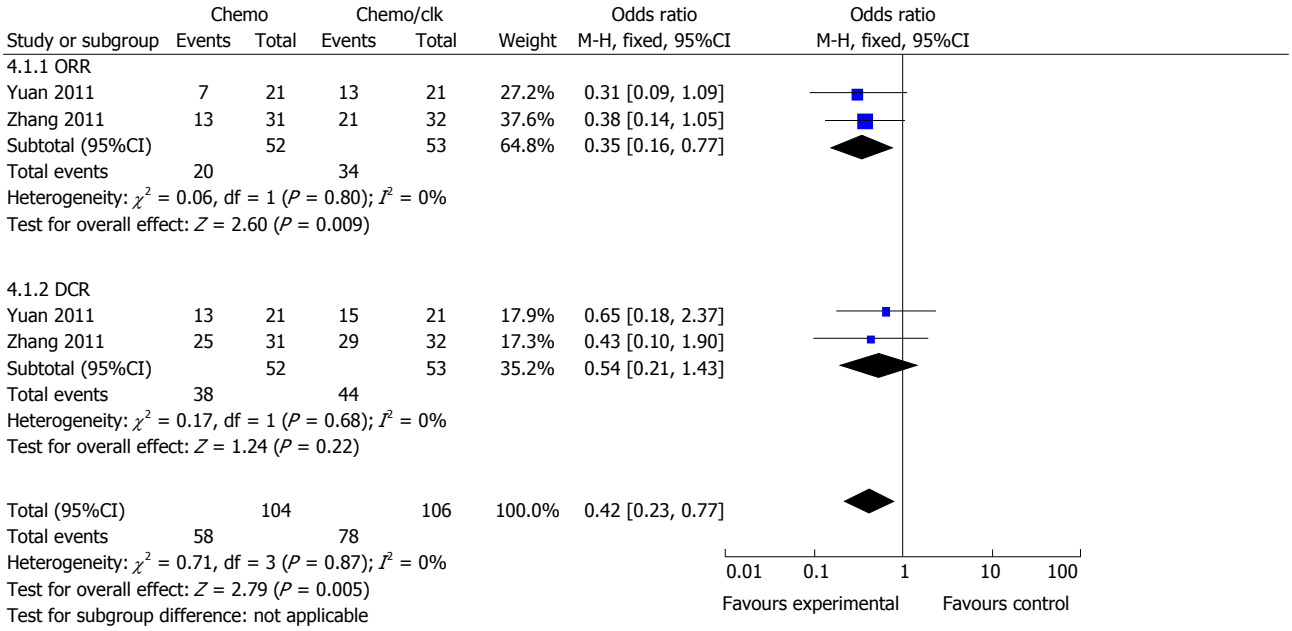


Figure 4 Comparison of objective response rate and disease control rate following treatment with chemo-dendritic cell-cytokine-induced killer cells and chemo-alone. The fixed effects meta-analysis model (Mantel-Haenszel method) was used in this analysis. ORR: Objective response rate; DCR: Disease control rate.

$CD4^+$ cells (95%CI: -9.97--3.62, $P < 0.0001$) which were significantly changed between the two groups (Figure 5), while -9.82 for $CD3^+$ cells (95%CI: -22.36-2.73, $P = 0.13$), 1.44 for $CD8^+$ cells (95%CI: -8.90-11.78, $P = 0.78$) and -0.71 for $CD4^+CD8^+$ cells (95%CI: -1.44-0.02, $P = 0.06$) did not differ. Overall, most of the selected T cell subsets were significantly increased after treatment with DC-CIK ($P < 0.00001$) (Figure 5).

DISCUSSION

Colon cancer is the third most common cancer in the world and more than half of patients diagnosed with colon cancer will eventually die as a result of cancer-associated complications. Therefore, there is an urgent need for improvements in adjuvant therapies, as well as improved treatment options for metastatic disease.

The first clinical trial of CIK therapy was reported in 1999 by Schmidt-Wolf, while the first clinical studies of DC-based vaccines were described in 1973 by Steinman and Cohn. Additional studies have demonstrated that CIK cells and DC vaccine therapies have anti-tumor effects. Despite the drawbacks associated with *in vitro* cell manipulation and upscaling, several approaches have been assessed in clinical cancer treatment. The use of DC vaccine, LAK3 cells, CTLs, CIK cells, and tumor infiltrating lymphocytes have been well studied, and additional trials are ongoing. Increasing information on the clinical anti-tumor activity of DC-CIK cells is available from autologous therapy trials and some systematic reviews yielded several findings. A meta-analysis of DC-based tumor vaccination in prostate and renal cell cancers[27], adoptive immunotherapy in postoperative hepatocellular carcinoma (HCC)[28] and CIK cell therapy in patients with HCC and solid carcinomas[29] confirmed that immunotherapy is a safe and feasible treatment option for cancer patients. However, there are also some limitations. For instance, cancers that escape host immune surveillance are generally more difficult to cure. Furthermore, it is often difficult to obtain the necessary numbers of cytotoxic cells that are required for effective tumor control.

A growing body of knowledge on tumor immunosurveillance and loss thereof has contributed to the refinement of anti-tumor immunotherapy. The aim of our meta-analysis was to determine whether an association exists between DC-CIK cell therapy combined with chemotherapy and chemotherapy alone in terms of 1, 2 and 3-year overall survival, 1, 2 and 3-year disease-free survival, ORR and immune indices in colon cancer. This study was also designed to elucidate whether DC-CIK cell therapy can enhance the therapeutic efficacy of chemotherapy in colon cancer.

First, our analysis showed that DC-CIK therapies were associated with significantly prolonged 1-year OS (OR = 0.23; 95%CI: 0.11-0.48, $P < 0.0001$), 2-year OS (OR = 0.42; 95%CI: 0.22-0.81, $P = 0.009$) and 3-year OS (OR = 0.43; 95%CI: 0.25-0.74, $P = 0.002$). There was no 5-year OS data in these studies. Our results, based on prospective studies showed that DC-CIK therapies combined with chemotherapy improved survival in colon cancer patients compared with chemotherapy alone. In contrast, DC-CIK therapies plus chemotherapy did not improve survival in HCC patients. In that analysis, 3-year or 5-year survival was not statistically different with CIK therapies[30]. In addition, different tumor stages (Ⅰ to Ⅳ) were included in the trials and some trial data of 1-year, 2-year and 3-year survival rate were not adequately provided. Thus, when we collected the data, survival at 2

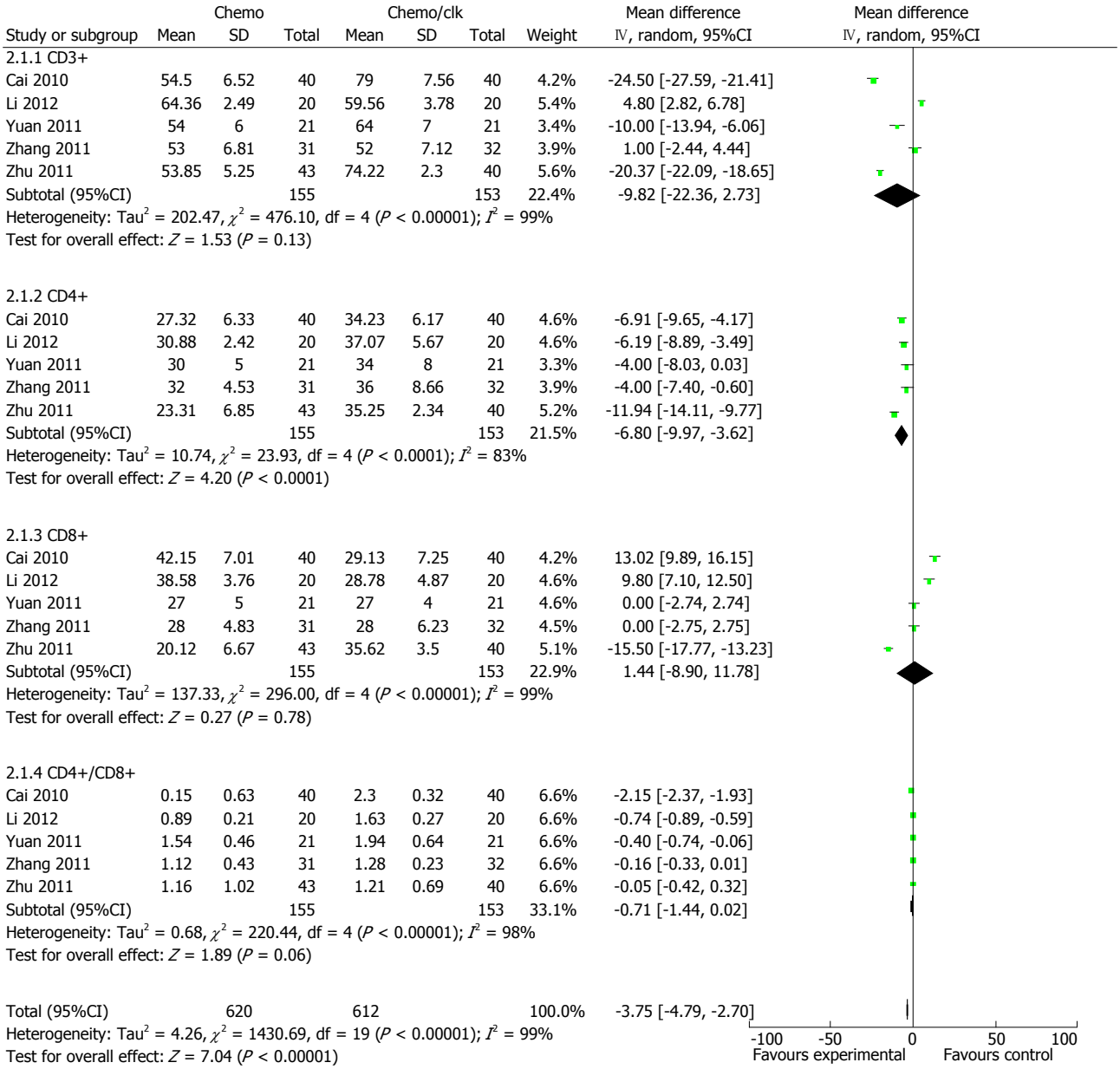


Figure 5 Forest plot of immunophenotype assessment. Data from patients after chemo-dendritic cell-cytokine-induced killer cell treatment and chemo-alone treatment. The random effects meta-analysis model (Mantel-Haenszel method) was used in this analysis.

years (76%) included five trials, while survival at 3 years (80%) included three trials, which had an impact on the results.

Second, our analysis of DFS demonstrated that patients receiving DC-CIK cell therapy had better DFS compared with patients in the chemotherapy alone group, 1-year DFS (OR = 0.24; 95%CI: 0.12-0.49, $P < 0.0001$), 2-year DFS (OR = 0.41; 95%CI: 0.23-0.71, $P = 0.002$) and 3-year DFS (OR = 0.50; 95%CI: 0.29-0.88, $P = 0.02$). GOLFIG represents a chemo-immunotherapeutic regimen which includes the standard poly-chemotherapy FOLFOX (5-fluorouracil, FU, leucovorin and oxaliplatin) plus gemcitabine and an immunoadjuvant treatment with subcutaneous injections of GM-CSF and low-dose IL-2, which showed high response rates and disease control rates as well as prolonged time to progression in colon cancer patients[31-34]. It is known that DC-CIK immunotherapy promotes the secretion of many cytokine factors, including IL-2, thus our promising results, showing prolonged OS and DFS, provide evidence for the potential application of DC-CIK (adoptive cell therapy) combined with chemotherapy in colon cancer. Furthermore, there may be some interactions between different chemotherapy regimens and immunotherapy, therefore, we analyzed the different chemotherapy categories and found no significant impact on the results. Thus, immunotherapy plays a role in the treatment of colon cancer. It should be noted that the DFS analysis only included two trials for each endpoint, therefore, a larger number of trials are required to confirm these findings.

Third, the analysis of ORR demonstrated that the ORR increased significantly in the DC-CIK group with

an OR of 0.35 (95%CI: 0.16-0.77, $P = 0.009$) compared with the non-DC-CIK group. However, the DCR did not significantly differ from the chemotherapy alone group (OR 0.54; 95%CI: 0.21-1.43, $P = 0.22$) (Figure 4). In an ongoing pilot study, sentinel node-derived lymphocytes were infused in colon cancer patients. Clinical responses were seen in stage Ⅳ colon cancer patients and complete remission (CR) was observed in four patients who received a significantly larger numbers of T cells than patients with SD and PR[35]. Therefore, in our selected studies, the difference in the number of transfused T cells in the different groups of patients, which would lead to the clinical response of DCR was not obvious.

Fourth, the human immune response against a tumor is mainly dependent on cellular immunity. The ratios of T-lymphocyte subsets in the peripheral blood are usually distorted in tumor patients. In the present analysis, the percentages of $CD4^+$ T cells were significantly increased in the DC-CIK group compared with the chemotherapy group ($P < 0.05$). We demonstrated that DC-CIK cells were suitable for enhancing the antitumor activity in colon cancer patients. Furthermore, the tumor-specific responses generation were potent, long-lasting, and required T cells[36]. The therapeutic efficacy of immunotherapies generally correlates with the generation of strong antigen-specific T- and B-cell responses, and augmentation of such responses may increase the overall potency of immunotherapies. $CD8^+$ cell ($P = 0.78$) percentages did not differ between the two groups after treatment. It is known that the mere presence of tumor-specific $CD8^+$ T cells (cytotoxic T cells) in the peripheral blood is not correlated with improved clinical outcome. In contrast, several studies indicated a correlation between the number of tumor infiltrating $CD8^+$ T lymphocytes (TILs) and an improved prognosis in colorectal cancer[37], but not in other cancers, such as HCC[38]. Thus, compared to chemotherapy alone, combining DC-CIK immunotherapy with chemotherapy has greater precision to seek and kill tumor cells and helps to increase the sensitivity of cancer cells to chemotherapy, thus providing hope for the treatment of colorectal cancer.

Limitations of the study

Our meta-analysis has limitations that affect interpretation of the results. First, all seven trials included in the analysis were conducted in China, and published only in the Chinese language. However, it should be noted that on the website http://www.immunitynet.com/coloncancer.asp, it has been demonstrated that there are many successful cases of patients with colon cancer treated with immunotherapy. A clinical trial performed by Guangxi Medical University was registered in http://clinicaltrials.gov/ (NCT01839539). Schmidt-Wolf *et al*[19] showed that seven colon cancer patients responded to CIK cell therapy and we excluded this Germany study from our selected trials. In addition, a successful case treated with CIK cells alone was reported by Sun Yatsen University Cancer Center and published in English[39]. Furthermore, the seven trials included a total of 533 patients, and none of the trials had more than 100 patients per arm. Thus, a larger sample size including more patients in all groups is needed. The follow-up period was also not sufficiently long. Some of the studies did not even report the follow-up time, tumor size, or background colon diseases. Moreover, patient information was limited in some cases.

The reliability of this systemic review might also be influenced by other factors. For example, not all of the included studies reported clinic random allocation concealment, thus, the meta-analysis may have distribution and implementation bias. We summarized the data from the published results, which will have introduced bias across the studies. Clinical studies with DC-CIK cells are still in their infancy and only involve a relatively small number of patients in most of these studies. The relatively robust and simple cell culture procedures to expand DC-CIK cells have enabled the approach of adoptive cellular immunotherapy to be widely studied. Based on the encouraging experimental and clinical evidence currently available, randomized clinical trials are justifiable and should be performed under stringent compliance with the CONSORT principles. This will involve a large number of patients in order to demonstrate statistical significance for a modest degree of outcome superiority. Such studies are urgently needed in order to provide unequivocal evidence of the clinical usefulness of immunotherapy.

Collectively, our analysis demonstrates that DC-CIK therapy can result in enhanced survival and improved clinical responses in colorectal patients. We also found that these DC-CIK-mediated improvements typically correspond with enhanced immune function. Thus, DC-CIK cells can enhance the therapeutic efficacy of chemotherapy in colon cancer patients. Hence, the efficacy of this therapy lies in its possible application as a promising adjuvant therapy for colon cancer. However, further development of this immunotherapy is needed.

COMMENTS

Background

Colorectal cancer is the third most commonly diagnosed cancer in humans. However, 25% of patients who present with metastatic disease have a five-year survival of only 10%. In recent years there has been great interest in cancer immunotherapy, which has the potential to control metastatic disease, prolong time to recurrence, and ultimately serve as a preventive measure. However, clinical studies on dendritic cell (DC)-cytokine-induced killer (CIK) cells are still in their infancy.

Research frontiers

The bedside medication administration of Sipuleucel-T (Provenge, Dendreon) was the first-in class therapeutic autologous vaccine to be approved for the treatment of men with asymptomatic or minimally symptomatic castrate-resistant metastatic prostate cancer in the spring of 2010. A phase Ⅰ-Ⅱ carcinoembryonic antigen-loaded DC vaccine trial in patients with colon cancer is ongoing (www.clinicaltrial.gov id: NCT 01219348). Based on findings in the tumor microenvironment and cancer stem cells in cancer therapy, immunotherapy is worth further investigation.

Innovations and breakthroughs

Over the past few years, advances in their understanding of the immune system, improved design of cancer immunotherapy clinical trials and compliance of manufacturing processes have provided opportunities to significantly improve

the efficacy and safety of treatment. However, clinical studies on DC-CIK cells have not achieved a clear consensus on how they may be best optimized. Therefore, the authors performed a systematic review and meta-analysis of clinical trials to assess the therapeutic efficacy of DC-CIK cells combined with chemotherapy in colon cancer. The pooled analysis was performed using the data from random or fixed-effect models. The overall analysis showed a significant one-year, two-year and three-year survival (overall survival) benefit with DC-CIK immunotherapy and chemotherapy. One-year, two-year and three-year disease-free survival rates were also improved after treatment with the combination of DC-CIK immunotherapy and chemotherapy. An improvement in overall response rate was also observed in patients who received additional DC-CIK cell therapy. Furthermore, the analysis of T-lymphocyte subsets in peripheral blood indicated that the number of $CD4^+$ T cells significantly increased in the DC-CIK plus chemotherapy group, implying enhanced immunological responses for anti-tumor regulation. There were no alterations in the number of $CD3^+$, $CD8^+$ and $CD4^+CD8^+$ T cells following DC-CIK treatment, suggesting that T cell-mediated cytotoxicity was not aggravated. In all, the combination of DC-CIK immunotherapy and chemotherapy was superior in prolonging survival time and enhancing immunological responses.

Applications

The analysis demonstrated that the efficacy of DC-CIK therapy lies in its possible application as a promising adjuvant therapy for colon cancer.

Terminology

DCs constitute a unique subset of extremely efficient antigen-presenting cells. They were first described in 1973 by Steinman and Cohn. Steinman received the 2011 Nobel Prize in Physiology or Medicine for the discovery of the dendritic cell and its role in adaptive immunity. CIK cells are non-major histocompatibility complex-restricted $CD3^+CD56^+$ T cells. They were first described as having a marked ability to proliferate and an increased superiority over lymphokine-activated killer cells in cytolytic activity against cancer by Schmidt Wolf *et al*. Adoptive immunotherapy is used in the treatment of cancer in which an individual's own white blood cells are coupled with a naturally produced growth factor to enhance their cancer-fighting capacity and holds great promise in the scenario of potential new approaches for the treatment of solid tumors that are refractory to conventional therapies.

Peer review

In this manuscript the authors investigated whether autologous DC-CIK therapy was able to improve the therapeutic efficacy of chemotherapy in colon cancer. They conducted a systematic review of published papers from several different sources. Their findings support that the combination of the DC-CIK immunotherapy and chemotherapy has superiority in prolonging the survival time and enhancing immunological responses. In recent years there has been great interest in cancer immunotherapy, which has the potential of controlling metastatic disease, prolonging time to recurrence, and ultimately serving as a preventive measure. The study is well performed and the manuscript is clear and convincing.

REFERENCES

1 **Siegel R**, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin* 2013; **63**: 11-30 [PMID: 23335087 DOI: 10.3322/caac.21166]

2 **Yang VW**, Lewis J, Wang TC, Rustgi AK. Colon cancer: an update and future directions. *Gastroenterology* 2010; **138**: 2027-2028 [PMID: 20226784 DOI: 10.1053/j.gastro]

3 **Yu Y**, Cho HI, Wang D, Kaosaard K, Anasetti C, Celis E, Yu XZ. Adoptive transfer of Tc1 or Tc17 cells elicits anti-tumor immunity against established melanoma through distinct mechanisms. *J Immunol* 2013; **190**: 1873-1881 [PMID: 23315072 DOI: 10.4049/jimmunol.1201989]

4 **Jäkel CE**, Hauser S, Rogenhofer S, Müller SC, Brossart P, Schmidt-Wolf IG. Clinical studies applying cytokine-induced killer cells for the treatment of renal cell carcinoma. *Clin Dev Immunol* 2012; **2012**: 473245 [PMID: 23193418 DOI: 10.1155/2012/473245]

5 **Kharfan-Dabaja MA**, Hamadani M, Reljic T, Nishihori T, Bensinger W, Djulbegovic B, Kumar A. Comparative efficacy of tandem autologous versus autologous followed by allogeneic hematopoietic cell transplantation in patients with newly diagnosed multiple myeloma: a systematic review and meta-analysis of randomized controlled trials. *J Hematol Oncol* 2013; **6**: 2 [PMID: 23289975 DOI: 10.1186/1756-8722-6-2]

6 **Chow KK**, Naik S, Kakarla S, Brawley VS, Shaffer DR, Yi Z, Rainusso N, Wu MF, Liu H, Kew Y, Grossman RG, Powell S, Lee D, Ahmed N, Gottschalk S. T cells redirected to EphA2 for the immunotherapy of glioblastoma. *Mol Ther* 2013; **21**: 629-637 [PMID: 23070117 DOI: 10.1038/mt.2012.210]

7 **Li Z**. Potential of human γδ T cells for immunotherapy of osteosarcoma. *Mol Biol Rep* 2013; **40**: 427-437 [PMID: 23065272 DOI: 10.1007/s11033-012-2077-y]

8 **Iwai K**, Soejima K, Kudoh S, Umezato Y, Kaneko T, Yoshimori K, Tokuda H, Yamaguchi T, Mizoo A, Setoguchi Y, Kamigaki T, Fujimoto K, Goto S. Extended survival observed in adoptive activated T lymphocyte immunotherapy for advanced lung cancer: results of a multicenter historical cohort study. *Cancer Immunol Immunother* 2012; **61**: 1781-1790 [PMID: 22422103]

9 **Higano CS**, Small EJ, Schellhammer P, Yasothan U, Gubernick S, Kirkpatrick P, Kantoff PW. Sipuleucel-T. *Nat Rev Drug Discov* 2010; **9**: 513-514 [PMID: 20592741 DOI: 10.1038/nrd3220]

10 **Gardner TA**, Elzey BD, Hahn NM. Sipuleucel-T (Provenge) autologous vaccine approved for treatment of men with asymptomatic or minimally symptomatic castrate-resistant metastatic prostate cancer. *Hum Vaccin Immunother* 2012; **8**: 534-539 [PMID: 22832254 DOI: 10.4161/hv.19795]

11 **Hodi FS**, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, Akerley W, van den Eertwegh AJ, Lutzky J, Lorigan P, Vaubel JM, Linette GP, Hogg D, Ottensmeier CH, Lebbé C, Peschel C, Quirt I, Clark JI, Wolchok JD, Weber JS, Tian J, Yellin MJ, Nichol GM, Hoos A, Urba WJ. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010; **363**: 711-723 [PMID: 20525992 DOI: 10.1056/NEJMoa1003466]

12 **Robert C**, Thomas L, Bondarenko I, O'Day S, M D JW, Garbe C, Lebbe C, Baurain JF, Testori A, Grob JJ, Davidson N, Richards J, Maio M, Hauschild A, Miller WH, Gascon P, Lotem M, Harmankaya K, Ibrahim R, Francis S, Chen TT, Humphrey R, Hoos A, Wolchok JD. Ipilimumab plus dacarbazine for previously untreated metastatic melanoma. *N Engl J Med* 2011; **364**: 2517-2526 [PMID: 21639810 DOI: 10.1056/NEJMoa1104621]

13 **Lee JH**, Park MS, Chung IJ. Induction of 90K-specific Cytotoxic T Lymphocytes for Colon Cancer Immunotherapy. *Immune Netw* 2010; **10**: 206-211 [PMID: 21286381 DOI: 10.4110/in.2010.10.6.206]

14 **Mukherjee P**, Pathangey LB, Bradley JB, Tinder TL, Basu GD, Akporiaye ET, Gendler SJ. MUC1-specific immune therapy generates a strong anti-tumor response in a MUC1-tolerant colon cancer model. *Vaccine* 2007; **25**: 1607-1618 [PMID: 17166639]

15 **Inoda S**, Hirohashi Y, Torigoe T, Morita R, Takahashi A, Asanuma H, Nakatsugawa M, Nishizawa S, Tamura Y, Tsuruma T, Terui T, Kondo T, Ishitani K, Hasegawa T, Hirata K, Sato N. Cytotoxic T lymphocytes efficiently recognize human colon cancer stem-like cells. *Am J Pathol* 2011; **178**: 1805-1813 [PMID: 21435460 DOI: 10.1016/j.ajpath.2011.01.004]

16 **Iwata-Kajihara T**, Sumimoto H, Kawamura N, Ueda R, Takahashi T, Mizuguchi H, Miyagishi M, Takeda K, Kawakami Y. Enhanced cancer immunotherapy using STAT3-depleted dendritic cells with high Th1-inducing ability and resistance to cancer cell-derived inhibitory factors. *J Immunol* 2011; **187**: 27-36 [PMID: 21632716 DOI: 10.4049/jimmunol.1002067]

17 **Lesterhuis WJ**, De Vries IJ, Schreibelt G, Schuurhuis DH, Aarntzen EH, De Boer A, Scharenborg NM, Van De Rakt M, Hesselink EJ, Figdor CG, Adema GJ, Punt CJ. Immunogenicity of dendritic cells pulsed with CEA peptide or transfected with CEA mRNA for vaccination of colorectal cancer pa-

tients. *Anticancer Res* 2010; **30**: 5091-5097 [PMID: 21187495]
18 **Lu J**, Liu K, Zhao J, Zhao J, Ma J, Yang H, Huang Y, Qin Z, Bai R, Jiang L, Lv F, Li P, Yan W, Zhao M, Dong Z. VEGF-A not Ang2 mediates endothelial-like differentiation of immature DCs by ERK1/2 signaling in the microenvironment of human colon adenocarcinoma. *Int J Oncol* 2011; **38**: 1579-1588 [PMID: 21455573 DOI: 10.3892/ijo.2011.989]
19 **Schmidt-Wolf IG**, Finke S, Trojaneck B, Denkena A, Lefterova P, Schwella N, Heuft HG, Prange G, Korte M, Takeya M, Dorbic T, Neubauer A, Wittig B, Huhn D. Phase I clinical study applying autologous immunological effector cells transfected with the interleukin-2 gene in patients with metastatic renal cancer, colorectal cancer and lymphoma. *Br J Cancer* 1999; **81**: 1009-1016 [PMID: 10576658]
20 **Cai XY**, Xiong W, Li YF, Shen T. Clinical research of the treatment on patients of mid-low locally rectal cancer after-operation with cytokine induced killers. *Shiyong Aizheng Zazhi* 2010; **25**: 37-39 [DOI: 10.3969/j.issn.1001-5930.2010.01.011]
21 **Li S**, Li Y, Liang J, Liu XL. The study of clinical application of DC-CIK combined with chemotherapy on colon cancer. *Zhongguo Mianyixue Zazhi* 2012; **9**: 835-839 [DOI: 10.3969/j.issn.1000-484X.2012.09.016]
22 **Wei ZQ**, Yang JW, Chen LC, Zheng QH, Ying MG. A retrospective analysis of postsurgical treatment for rectal cancer by chemotherapy and radiotherapy combined with dentritic cells. *Fujian Yike Daxue Xuebao* 2009; **43**: 483-487 [DOI: 10.3969/j.issn.1672-4194.2009.06.013]
23 **Ying MG**, Wei ZQ, Yang JW, Chen LC, Zheng QH. Retrospective analysis of pos-operative chemo-radiotherapy combined with DC-CIK in the treatment of patients with colorectal cancer. *Shiyong Aizheng Zazhi* 2010; **25**: 274-276 [DOI: 10.3969/j.issn.1001-5930.2010.03.016]
24 **Zhang J**, Geng J, Han ZX, Gao XY. Chemotherapy combined with CIK/DC cells in the treatment of advanced colorectal cancer: a clinical observation. *Xuzhou Yixueyuan Xuebao* 2011; **7**: 457-459 [DOI: 10.3969/j.issn.1000-2065.2011.07.008]
25 **Zhu Y**, Liu JQ, Zhang NZ, Chen FX, Chen L, Zhang S, Yang WaY, Zhou ZH, Xu YM. Clinical effects of treatment with comprehensive multipe autologous immune cells in patients with coclorectal carcinoma. *Xuzhou Yixueyuan Xuebao* 2011; **9**: 631-636 [DOI: 10.3969/j.issn.1000-2065.2011.09.018]
26 **Yuan JH**, Peng DW, Li JW, Wang MQ. Clinical research of dendritic cells combined with cytokine induced killer cells therapy for advanced colorectal cancer. *Zhongguo Quanke Yixue* 2011; **36**: 4139-4141 [DOI: 10.3969/j.issn.1007-9572.2011.36.007]
27 **Draube A**, Klein-González N, Mattheus S, Brillant C, Hellmich M, Engert A, von Bergwelt-Baildon M. Dendritic cell based tumor vaccination in prostate and renal cell cancer: a systematic review and meta-analysis. *PLoS One* 2011; **6**: e18801 [PMID: 21533099 DOI: 10.1371/journal.pone.0018801]
28 **Xie F**, Zhang X, Li H, Zheng T, Xu F, Shen R, Yan L, Yang J, He J. Adoptive immunotherapy in postoperative hepatocellular carcinoma: a systemic review. *PLoS One* 2012; **7**: e42879 [PMID: 22916174 DOI: 10.1371/journal.pone.0042879]
29 **Ma Y**, Zhang Z, Tang L, Xu YC, Xie ZM, Gu XF, Wang HX. Cytokine-induced killer cells in the treatment of patients with solid carcinomas: a systematic review and pooled analysis. *Cytotherapy* 2012; **14**: 483-493 [PMID: 22277010 DOI: 10.3109/14653249]
30 **Ma Y**, Xu YC, Tang L, Zhang Z, Wang J, Wang HX. Cytokine-induced killer (CIK) cell therapy for patients with hepatocellular carcinoma: efficacy and safety. *Exp Hematol Oncol* 2012; **1**: 11 [PMID: 23210562 DOI: 10.1186/2162-3619-1-11]
31 **Correale P**, Cusi MG, Tsang KY, Del Vecchio MT, Marsili S, Placa ML, Intrivici C, Aquino A, Micheli L, Nencini C, Ferrari F, Giorgi G, Bonmassar E, Francini G. Chemo-immunotherapy of metastatic colorectal carcinoma with gemcitabine plus FOLFOX 4 followed by subcutaneous granulocyte macrophage colony-stimulating factor and interleukin-2 induces strong immunologic and antitumor activity in metastatic colon cancer patients. *J Clin Oncol* 2005; **23**: 8950-8958 [PMID: 16061910]
32 **Correale P**, Tagliaferri P, Fioravanti A, Del Vecchio MT, Remondo C, Montagnani F, Rotundo MS, Ginanneschi C, Martellucci I, Francini E, Cusi MG, Tassone P, Francini G. Immunity feedback and clinical outcome in colon cancer patients undergoing chemoimmunotherapy with gemcitabine + FOLFOX followed by subcutaneous granulocyte macrophage colony-stimulating factor and aldesleukin (GOLFIG-1 Trial). *Clin Cancer Res* 2008; **14**: 4192-4199 [PMID: 18593999 DOI: 10.1158/1078-0432.CCR-07-5278]
33 **Correale P**, Rotundo MS, Del Vecchio MT, Remondo C, Migali C, Ginanneschi C, Tsang KY, Licchetta A, Mannucci S, Loiacono L, Tassone P, Francini G, Tagliaferri P. Regulatory (FoxP3+) T-cell tumor infiltration is a favorable prognostic factor in advanced colon cancer patients undergoing chemo or chemoimmunotherapy. *J Immunother* 2010; **33**: 435-441 [PMID: 20386463 DOI: 10.1097/CJI.0b013e3181d32f01]
34 **Ellebaek E**, Andersen MH, Svane IM, Straten PT. Immunotherapy for metastatic colorectal cancer: present status and new options. *Scand J Gastroenterol* 2012; **47**: 315-324 [PMID: 22214467 DOI: 10.3109/00365521.2012.640831]
35 **Karlsson M**, Marits P, Dahl K, Dagöö T, Enerbäck S, Thörn M, Winqvist O. Pilot study of sentinel-node-based adoptive immunotherapy in advanced colorectal cancer. *Ann Surg Oncol* 2010; **17**: 1747-1757 [PMID: 20119674 DOI: 10.1245/s10434-010-0920-8]
36 **Liu Z**, Noh HS, Chen J, Kim JH, Falo LD, You Z. Potent tumor-specific protection ignited by adoptively transferred CD4+ T cells. *J Immunol* 2008; **181**: 4363-4370 [PMID: 18768895]
37 **Koch M**, Beckhove P, Op den Winkel J, Autenrieth D, Wagner P, Nummer D, Specht S, Antolovic D, Galindo L, Schmitz-Winnenthal FH, Schirrmacher V, Büchler MW, Weitz J. Tumor infiltrating T lymphocytes in colorectal cancer: Tumor-selective activation and cytotoxic activity in situ. *Ann Surg* 2006; **244**: 986-992; discussion 992-993 [PMID: 17122624]
38 **Zhao M**, Wu PH, Zeng YX, Xia JC, Zhang FJ, Xian LJ, Zhang YP, Zhou K, Fan WJ, Zhang L, Gao F, Zhou QM. [Cytokine-induced killer cell fusion to lower recurrence of hepatocellular carcinoma after transcatheter arterial chemoembolization sequentially combined with radiofrequency ablation: a randomized trial]. *Zhonghua Yixue Zazhi* 2006; **86**: 1823-1828 [PMID: 17054858]
39 **Xia LP**, Wu PH, Xia JC, Zhang B, Guan ZZ, Wan DS, Guo GF, Zeng YX. One patient with metastastic colorectal cancer successfully treated by combination of targeted agents after failure of chemotherapy. *Chin J Cancer* 2010; **29**: 1023-1028 [PMID: 21114924]

P- Reviewers: D'Orazi G, Padin-Iruegas ME, Yeh JY
S- Editor: Gou SX **L- Editor**: Webster JR **E- Editor**: Liu XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.1107

World J Gastroenterol 2014 January 28; 20(4): 1107-1113
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

META-ANALYSIS

Meta-analysis of immunohistochemical expression of hypoxia inducible factor-1α as a prognostic role in gastric cancer

Shuang Lin, Rui Ma, Xue-Yong Zheng, Hong Yu, Xiao Liang, Hui Lin, Xiu-Jun Cai

Shuang Lin, Xue-Yong Zheng, Hong Yu, Xiao Liang, Hui Lin, Xiu-Jun Cai, Department of General Surgery, Sir Run Run Shaw Hospital, College of Medicine, Institute of Minimally Invasive Surgery, Zhejiang University, Hangzhou 310016, Zhejiang Province, China
Rui Ma, Department of Surgery, Zhejiang University Hospital, Zhejiang University, Hangzhou 310027, Zhejiang Province, China
Author contributions: Lin S and Cai XJ wrote the paper and performed the research; Cai XJ and Ma R designed the study; Zheng XY and Yu H collected the data; Liang X and Lin H performed the literature search and retrieved the data; and Cai XJ analyzed the data.
Correspondence to: Xiu-Jun Cai, MD, Department of General Surgery, Sir Run Run Shaw Hospital, College of Medicine, Institute of Minimally Invasive Surgery, Zhejiang University, No. 3, East Qingchun Road, Hangzhou 310016, Zhejiang Province, China. cxjzu@hotmail.com
Telephone: +86-571-86006605 Fax: +86-571-86006276
Received: September 25, 2013 Revised: October 26, 2013
Accepted: November 12, 2013
Published online: January 28, 2014

Abstract

AIM: To conduct a meta-analysis to evaluate the prognostic role of hypoxia inducible factor-1α (HIF-1α) expression in gastric cancer.

METHODS: The PubMed, EMBASE, and Web of Science databases were searched systematically for all articles published in English before August, 2013. Pooled effect was calculated from the available data to evaluate the association between HIF-1α expression and 5-year overall survival and tumor clinicopathological features in gastric cancer patients. Pooled odds ratios (ORs) with 95%CIs were calculated using either a fixed-effects or a random-effects model.

RESULTS: Nine studies matched the selection criteria, which reported on 1103 subjects, 548 of whom had HIF-1α positive expression (50%). This meta-analysis indicated that HIF-1α positive expression in gastric cancer correlated with lower 5-year overall survival (OR = 0.36; 95%CI: 0.21-0.64), worse tumor differentiation (OR = 0.38; 95%CI: 0.23-0.64), deeper invasion (OR = 0.42; 95%CI: 0.32-0.57), higher rates of lymph node metastasis (OR = 2.23; 95%CI: 1.46-3.40), lymphatic invasion (OR = 2.50; 95%CI: 1.46-4.28), and vascular invasion (OR = 1.80; 95%CI: 1.29-2.51), and higher TNM stage (Ⅲ + Ⅳ) (OR = 0.31; 95%CI: 0.15-0.60).

CONCLUSION: HIF-1α positive expression indicates a poor prognosis for patients with gastric cancer. Further studies are required to confirm these results.

Key words: Hypoxia inducible factor-1α; Gastric cancer; 5-year overall survival; Clinicopathological features; Meta-analysis

Core tip: We conducted a meta-analysis to evaluate the correlation between hypoxia inducible factor-1α (HIF-1α) expression and clinical outcome in gastric cancer patients, and reported that HIF-1 positive expression indicates a poor prognosis for patients with gastric cancer. This is the first comprehensive and detailed meta-analysis to assess the association of HIF-1α positive expression with 5-year overall survival and tumor clinicopathological features for gastric cancer patients. We believed that the results will provide useful information for clinical decision-making regarding gastric cancer.

Lin S, Ma R, Zheng XY, Yu H, Liang X, Lin H, Cai XJ. Meta-analysis of immunohistochemical expression of hypoxia inducible factor-1α as a prognostic role in gastric cancer. *World J Gastroenterol* 2014; 20(4): 1107-1113 Available from: URL: http://

www.wjgnet.com/1007-9327/full/v20/i4/1107.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.1107

INTRODUCTION

Gastric cancer is one of the most common cancers worldwide. Although the prognosis of gastric cancer has improved due to early diagnosis, radical operation, and the development of adjuvant therapy, patients with gastric cancer still have a poor prognosis[1,2]. The main prognostic factors for gastric cancer are clinicopathological features of the disease, including tumor differentiation, depth of invasion, lymph node metastasis and stage. However, the prognostic factors do not fully predict individual clinical outcome. As a result, there is great interest in finding better markers to identify patients with a poor prognosis at the time of diagnosis[3,4]. Hypoxia inducible factor-1 (HIF-1) is a basic helix-loop-helix transcription factor composed of HIF-1α and HIF-1β subunits; and HIF-1α determines HIF-1 activity[5]. Increased evidence has revealed that HIF-1α positive expression was associated with an unfavorable prognosis in many kinds of cancer[6,7].

One published meta-analysis has reported that HIF-1α in Asian patients was associated with poor overall survival (OS), but not with disease free survival (DFS)[8]. However, there have been no data about tumor clinicopathological features, which were known to provide useful information for tumor prognosis. Since that meta-analysis only included several studies of low quality, the data reported were not sufficient to derive conclusions with regards to the OS and DFS. Given that several high-quality studies have been published recently, we reviewed the currently available evidence in the medical literature to determine the association between HIF-1α positive expression and 5-year overall survival of gastric cancer as well as common clinicopathological features, and to assess the significance of HIF-1α positive expression in the prediction of clinical outcome of gastric cancer.

MATERIALS AND METHODS

Study selection

The PubMed, EMBASE, and Web of Science databases were searched systematically for all articles published in English before August, 2013. The terms used for the search were: "HIF-1α" or "hypoxia-inducible factor-1α" and "Gastric Cancer" or "Gastric Neoplasm" or "Stomach Neoplasm".

Reference lists of all retrieved articles were also manually searched for additional studies. Two reviewers independently extracted the data from each study. All relevant text, tables, and figures were reviewed for data extraction. Discrepancies between the two reviewers were resolved by discussion and consensus.

Inclusion and exclusion criteria

Only studies in the English language were considered for inclusion. In addition, each study had to fulfill the following criteria: (1) patients with gastric cancer diagnosed by pathology; (2) studies that examined the relationship between HIF-1α and survival of gastric cancer; (3) studies that utilized immunohistochemistry to determine the expression of HIF-1α in paraffin-embedded surgical specimens; and (4) the most informative article when multiple articles were published by the same authors or groups.

Abstracts, letters, editorials and expert opinions, reviews without original data, case reports, and studies lacking a control group were excluded. The studies or data were also excluded for: (1) overlapping articles or duplicate data; (2) articles about cell lines or animals; (3) being impossible to extract the appropriate data from the published results; (4) conference records; (5) studies lacking information on survival; or (6) patients who had previous chemotherapy or radiotherapy.

Outcomes of interest and data extraction

We mainly aimed at evaluating the prognostic value of HIF-1α positive expression in gastric cancer patients regarding 5-year overall survival. Our second aim was to assess the association of HIF-1α positive expression with tumor clinicopathological features, such as tumor differentiation, depth of tumor invasion, lymph node metastasis, lymphatic invasion, vascular invasion, and tumor node metastasis (TNM) stage. Overall survival was measured from the date of medical resection to either the day of death or the day of the last follow-up visit.

Two reviewers independently extracted the following parameters from each study: (1) first author and year of publication; (2) study population characteristics; (3) number of subjects who were included in studies; and (4) 5-year overall survival and clinicopathological features.

Qualitative assessment

Quality assessment was performed with the Newcastle-Ottawa quality assessment scale (NOS).

Statistical analysis

The meta-analysis was performed using the Review Manager (RevMan) software, (version 5.2; Cochrane collaboration, http://ims.cochrane.org/revman/download). We analyzed dichotomous variables using estimation of odds ratio (OR) with 95%CI. The pooled effect was calculated using either a fixed-effects or a random-effects model. Heterogeneity between studies was evaluated using the χ^2 and I^2 tests, and we considered heterogeneity present if the I^2 statistic was $\geqslant$ 50%. $P < 0.05$ was considered significant. Assessment of publication bias for each of the pooled study groups was performed using a funnel plot.

RESULTS

Selection of trials

The initial search strategy retrieved 221 publications. After screening all titles, abstracts, and full texts, nine studies[9-17] met our entry criteria and were retrieved for more detailed evaluation (Figure 1). All nine studies were ret-

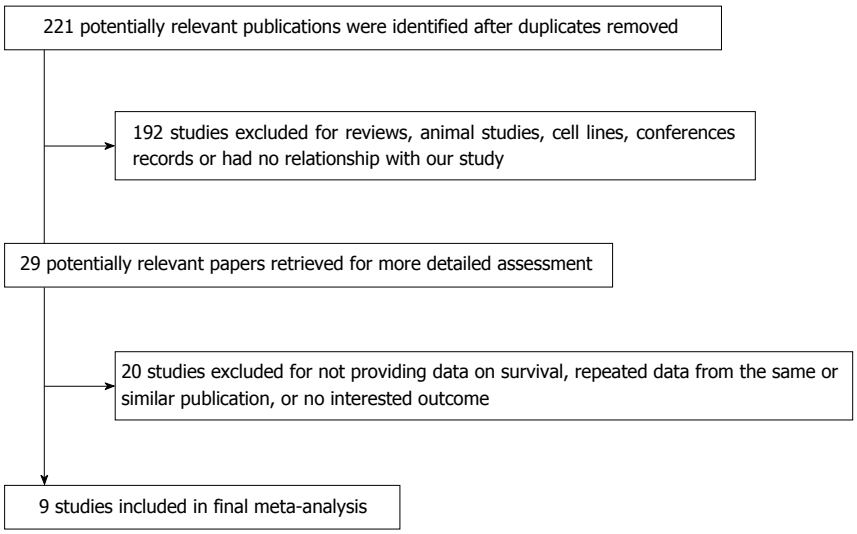


Figure 1 Flow diagram indicating the process of selecting articles for meta-analysis.

Table 1 Characteristics of the included studies

Ref.	Country of origin	Sample size (M/F, *n*)	Mean/median age (yr)	Study quality (points)	5-yr OS rate analysis	Expression rate
Zhan *et al*[9]	China	60 (38/22)	56.5	6/9	Reported	58.3%
Lu *et al*[10]	China	68 (43/25)	49.86	5/9	NR	52.9%
Isobe *et al*[11]	Japan	128 (91/37)	67.3	6/9	Reported	65.6%
Qiu *et al*[12]	China	188 (127/61)	57	6/9	Reported	54.6%
Oh *et al*[13]	South Korea	114 (67/47)	59	5/9	NR	15.8%
Kolev *et al*[14]	Japan	152 (110/42)	59.5	6/9	Reported	62.5%
Cabuk *et al*[15]	Turkey	51 (30/21)	63	4/9	NR	71%
Sumiyoshi *et al*[16]	Japan	216 (148/68)	65.2	5/9	NR	39.4%/85
Mizokami *et al*[17]	Japan	126 (83/41)	65.3	6/9	Reported	38.9%

Study quality was listed using the results of the Newcastle -Ottawa questionnaire. M: Male; F: Female; NR: Not reported; OS: Overall survival.

rospectively analyzed, and their characteristics are summarized in Table 1. Sample sizes ranged from 51 to 216, and the total number was 1103, 548 of whom had HIF-1α positive expression (50%). Of nine included studies, five provided data on 5-year overall survival. The studies were conducted in four countries (China, Japan, South Korea and Turkey).

Correlation between HIF-1α positive expression and 5-year overall survival

The 5-year overall survival was extracted from five studies. Meta-analysis indicated that patients with HIF-1α positive expression suffered with a lower 5-year overall survival (OR = 0.36; 95%CI: 0.21-0.64). The random effects model was used because of the heterogeneity (I^2 = 50.0%) (Figure 2A).

Correlation between HIF-1α positive expression and tumor clinicopathological features

Analysis of the pooled data showed that HIF-1α positive expression in gastric cancer was associated with biologically aggressive phenotypes such as tumor differentiation (OR = 0.38; 95%CI: 0.23-0.64; random effects model) (Figure 2B), depth of invasion (OR = 0.42; 95%CI: 0.32-0.57; fixed effects model) (Figure 2C), lymph node metastasis (OR = 2.23; 95%CI: 1.46-3.40; random effects model) (Figure 2D), lymphatic invasion (OR = 2.50; 95%CI: 1.46-4.28; random effects model) (Figure 2E), vascular invasion (OR = 1.80; 95%CI: 1.29-2.51; fixed effects model) (Figure 2F) and TNM stages Ⅲ + Ⅳ (OR = 0.31; 95%CI: 0.15-0.60; random effects model) (Figure 2G). In other words, the incidence of HIF-1α positive expression was significantly higher in the poorly differentiated and undifferentiated gastric cancer than in well and moderately differentiated types, and significantly lower in carcinomas in stages Ⅰ + Ⅱ than in stages Ⅲ + Ⅳ. HIF-1α positive expression was correlated with higher proportions of depth of invasion, lymphatic invasion, vascular invasion and lymph node metastasis.

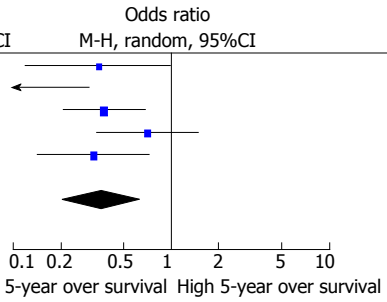
Publication bias

We used the inverted funnel plot to assess publication bias for all comparisons, and inspected its asymmetry visually. The shapes of the funnel plots showed a low potential for publication bias (Figure 3). Moreover, we used an influence analysis to evaluate the influence of a single study on the summary effect. The meta-analysis was not dominated by any individual study, and removing any study at a time made no difference.

A

Study or subgroup	HIF-1α positive Events	Total	HIF-1α negative Events	Total	Weight	Odds ratio M-H, random, 95%CI
1 Zhan H *et al*. 2013	12	35	15	25	17.1%	0.35 [0.12, 1.01]
3 Isobe T *et al*. 2013	53	84	43	44	6.6%	0.04 [0.01, 0.30]
4 Qiu MZ *et al*. 2011	36	110	44	78	28.9%	0.38 [0.21, 0.68]
6 Kolev Y *et al*. 2008	65	95	43	57	24.7%	0.71 [0.34, 1.48]
9 Mizokami, K *et al* 2006	29	49	63	77	22.8%	0.32 [0.14, 0.73]
Total (95%CI)		373		281	100.0%	0.36 [0.21, 0.64]
Total events	195		208			

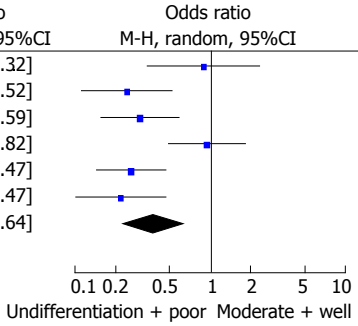
Heterogeneity: Tau^2 = 0.19; χ^2 = 7.94, df = 4 (P = 0.09); I^2 = 50%
Test for overall effect: Z = 3.53 (P = 0.0004)



B

Study or subgroup	HIF-1α positive Events	Total	HIF-1α negative Events	Total	Weight	Odds ratio M-H, random, 95%CI
2 Lu XX *et al*. 2013	17	36	16	32	13.6%	0.89 [0.34, 2.32]
3 Isobe T *et al*. 2013	25	84	28	44	16.1%	0.24 [0.11, 0.52]
4 Qiu MZ *et al*. 2011	20	110	33	78	17.7%	0.30 [0.16, 0.59]
6 Kolev Y *et al*. 2008	47	95	29	57	17.8%	0.95 [0.49, 1.82]
8 Sumiyoshi. Y *et al* 2006	23	85	77	131	18.8%	0.26 [0.14, 0.47]
9 Mizokami, K *et al* 2006	18	49	56	77	16.1%	0.22 [0.10, 0.47]
Total (95%CI)		459		419	100.0%	0.38 [0.23, 0.64]
Total events	150		239			

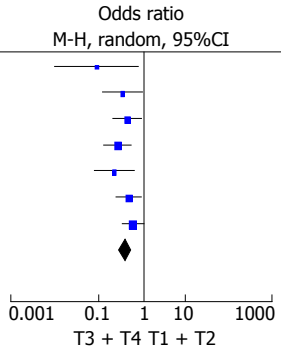
Heterogeneity: Tau^2 = 0.29; χ^2 = 15.84, df = 5 (P = 0.007); I^2 = 68%
Test for overall effect: Z = 3.61 (P = 0.0003)



C

Study or subgroup	HIF-1α positive Events	Total	HIF-1α negative Events	Total	Weight	Odds ratio M-H, random, 95%CI
1 Zhan H *et al*. 2013	1	35	6	25	5.0%	0.09 [0.01, 0.83]
2 Lu XX *et al*. 20139	8	36	14	32	8.5%	0.37 [0.13, 1.05]
3 Isobe T *et al*. 2013	32	84	25	44	15.0%	0.47 [0.22, 0.98]
4 Qiu MZ *et al*. 2011	15	110	28	78	20.9%	0.28 [0.14, 0.58]
5 Oh SY *et al*. 2008	7	18	70	96	10.0%	0.24 [0.08, 0.67]
6 Kolev Y *et al*. 2008	41	95	34	57	17.8%	0.51 [0.26, 1.00]
8 Sumiyoshi. Y *et al* 2006	45	85	84	131	22.9%	0.63 [0.36, 1.10]
Total (95%CI)		463		463	100.0%	0.42 [0.32, 0.57]
Total events	149		261			

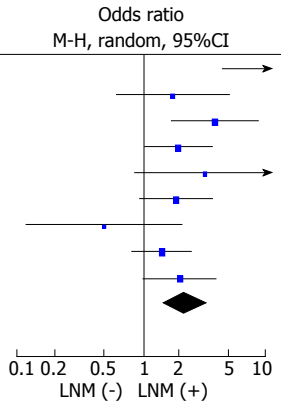
Heterogeneity: χ^2 = 6.68, df = 6 (P = 0.35); I^2 = 10%
Test for overall effect: Z = 5.72 (P < 0.00001)



D

Study or subgroup	HIF-1α positive Events	Total	HIF-1α negative Events	Total	Weight	Odds ratio M-H, random, 95%CI
1 Zhan H *et al*. 2013	32	35	9	25	6.2%	18.96 [4.50, 79.87]
2 Lu XX *et al*. 2013	27	36	20	32	9.5%	1.80 [0.64, 5.09]
3 Isobe T *et al*. 2013	50	84	12	44	12.5%	3.92 [1.77, 8.67]
4 Qiu MZ *et al*. 2011	82	110	44	74	14.9%	2.00 [1.06, 3.76]
5 Oh SY *et al*. 2008	15	18	58	96	7.1%	3.28 [0.89, 12.08]
6 Kolev Y *et al*. 2008	50	95	21	57	14.2%	1.90 [0.97, 3.73]
7 Cabuk, D. *et al*. 2007	24	36	12	15	6.2%	0.50 [0.12, 2.12]
8 Sumiyoshi. Y *et al* 2006	49	85	63	131	16.2%	1.47 [0.85, 2.55]
9 Mizokami, K *et al* 2006	25	49	26	77	13.3%	2.04 [0.98, 4.25]
Total (95%CI)		548		551	100.0%	2.23 [1.46, 3.40]
Total events	354		265			

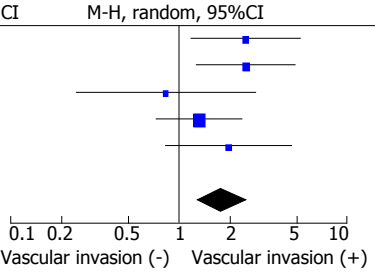
Heterogeneity: Tau^2 = 0.21; χ^2 = 17.46, df = 8 (P = 0.03); I^2 = 54%
Test for overall effect: Z = 3.72 (P = 0.0002)



E

Study or subgroup	HIF-1α positive Events	Total	HIF-1α negative Events	Total	Weight	Odds ratio M-H, random, 95%CI
3 Isobe T *et al*. 2013	55	84	19	44	16.8%	2.50 [1.18, 5.27]
6 Kolev Y *et al*. 2008	58	95	22	57	20.8%	2.49 [1.27, 4.89]
7 Cabuk, D. *et al*. 2007	20	36	9	15	11.0%	0.83 [0.24, 2.84]
8 Sumiyoshi. Y *et al* 2006	29	85	37	131	37.3%	1.32 [0.73, 2.73]
9 Mizokami, K *et al* 2006	14	49	13	77	14.1%	1.97 [0.83, 4.65]
Total (95%CI)		349		324	100.0%	1.80 [1.29, 2.51]
Total events	176		100			

Heterogeneity: χ^2 = 4.29, df = 4 (P = 0.37); I^2 = 7%
Test for overall effect: Z = 3.46 (P = 0.0005)

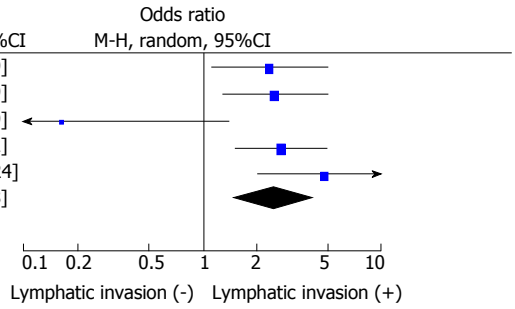


F

Study or subgroup	HIF-1α positive Events	Total	HIF-1α negative Events	Total	Weight	Odds ratio M-H, random, 95%CI
3 Isobe T *et al*. 2013	54	84	19	44	22.7%	2.37 [0.12, 4.99]
6 Kolev Y *et al*. 2008	66	95	27	57	24.5%	2.53 [1.28, 4.99]
7 Cabuk, D. *et al*. 2007	25	36	14	15	5.4%	0.16 [0.02, 1.39]
8 Sumiyoshi. Y *et al* 2006	61	85	63	131	27.3%	2.74 [1.53, 4.92]
9 Mizokami, K *et al* 2006	40	49	37	77	20.1%	4.80 [2.05, 11.24]
Total (95%CI)		349		324	100.0%	2.50 [1.46, 4.28]
Total events	246		160			

Heterogeneity: Tau^2 = 0.19; χ^2 = 8.59, df = 4 (P = 0.07); I^2 = 53%

Test for overall effect: Z = 3.33 (P = 0.0009)



G

Study or subgroup	HIF-1α positive Events	Total	HIF-1α negative Events	Total	Weight	Odds ratio M-H, random, 95%CI
1 Zhan H *et al*. 2013	3	35	16	25	11.5%	0.05 [0.01, 0.22]
2 Lu XX *et al*. 2013	10	36	16	22	13.8%	0.14 [0.04, 0.47]
4 Qiu MZ *et al*. 2011	41	110	56	78	19.9%	0.23 [0.12, 0.44]
5 Oh SY *et al*. 2008	7	18	60	96	15.4%	0.38 [0.14, 1.07]
6 Kolev Y *et al*. 2008	62	95	42	57	18.8%	0.67 [0.32, 1.39]
8 Sumiyoshi. Y *et al* 2006	45	85	80	131	20.7%	0.72 [0.41, 1.25]
Total (95%CI)		379		409	100.0%	0.31 [0.15, 0.60]
Total events	168		270			

Heterogeneity: Tau^2 = 0.50; χ^2 = 19.56, df = 5 (P = 0.002); I^2 = 74%

Test for overall effect: Z = 3.43 (P = 0.0006)

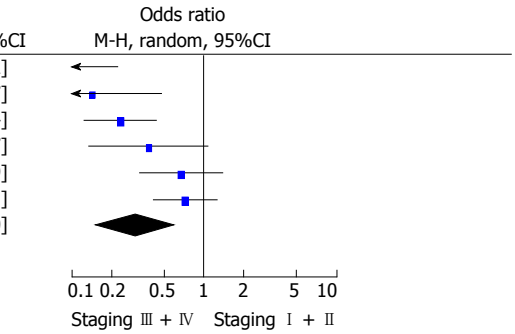


Figure 2 Forest plots. A: The results of the meta-analysis for 5-year overall survival; B: The results of the meta-analysis for tumor differentiation; C: The results of the meta-analysis for depth of invasion; D: The results of the meta-analysis for lymph node metastasis (LNM); E: The results of the meta-analysis for vascular invasion; F: The results of the meta-analysis for lymphatic invasion; G: The results of the meta-analysis for tumor node metastasis stage.

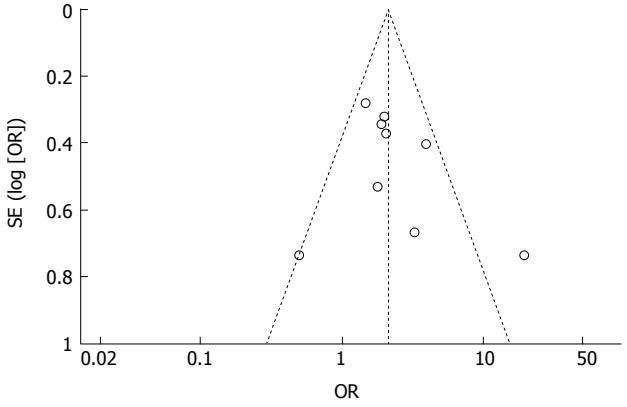


Figure 3 Funnel plot of studies of hypoxia inducible factor-1α positive expression in gastric cancer.

DISCUSSION

Meta-analysis has been traditionally applied and was mostly confined to randomized controlled trials (RCTs), and meta-analytical techniques using non-randomized controlled trials (NRCTs) might be a good method for use in some clinical settings in which either the number or the sample size of the RCTs is insufficient[18,19]. To our best knowledge, our study is the first comprehensive and detailed meta-analysis to assess the association of HIF-1α positive expression with 5-year overall survival and tumor clinicopathological features in gastric cancer patients. We believe that our results will provide useful information for clinical decision-making regarding gastric cancer.

Nowadays, many studies about the role of HIF-1α in tumors have already been conducted and the relationship between HIF-1α and tumors has been confirmed. HIF-1α plays a role in the tumor formation, progression and metastasis by activating genes which are related to regulation of angiogenesis, cell survival and metabolism[20-22]. Not all gastric cancers express HIF-1α and 548 (50%) of 1103 gastric cancer patients had HIF-1α positive expression in this meta-analysis. However, once gastric cancer cells acquire HIF-1α expression, they transform to have more aggressive and metastatic behavior. The meta-analysis about prognostic significance of HIF-1α has been studied in several cancers such as non-small cell lung cancer and hepatocellular carcinoma[5,23], and HIF-1α positive expression indicates a poor prognosis. In this study, we found that the 5-year overall survival in the HIF-1α positive group was significantly lower than that in the HIF-1α negative group. Thus, HIF-1α was a poor prognosis factor for gastric cancer patients.

Our result also demonstrated that HIF-1α positive expression was correlated with increased vascular invasion and lymphatic invasion. The presence of vascular invasion and lymphatic invasion may indicate increased biological aggressiveness and a greater possibility of systemic diffusion. As shown in previous studies, vascular invasion and lymphatic invasion were the main risk factors for tumor occurrence and had the close relation with tumor invasiveness[24,25]. Moreover, we analyzed the relationship between the expression of HIF-1α and clinicopathologic features of gastric cancer, and found that the expression of HIF-1α was related to higher proportions of poor tumor differentiation, deep invasion, lymph node metastasis and TNM stages Ⅲ + Ⅳ. This indicates that HIF-1α positive expression is closely

related to the poor biological behavior of gastric cancer.

There are several limitations to this meta-analysis, and consequently, the results should be interpreted with caution. First, the data came from NRCTs, and the overall level of clinical evidence was low. Abraham *et al*[26] had found that meta-analyses carried out on well designed NRCTs of surgical procedures were probably as accurate as those carried out on RCTs. Second, there was heterogeneity across studies. We applied a random-effects model to take variation between studies into consideration, and we believe that the heterogeneity would have had very limited influence. Third, reports in languages other than English were excluded. The risk of language bias had to be considered, but it may not result in any notable bias in the assessment of interventional effectiveness. Finally, publication bias was present in our analysis. The reason was that investigative groups might be more likely to report positive results, and that studies with significant outcomes are more likely to be published.

In conclusion, the results of this meta-analysis of 1103 patients showed that HIF-1α positive expression was associated with poor 5-year overall survival and clinicopathological features in patients with gastric cancer. Moreover, HIF-1α positive expression could be a useful prognostic marker for gastric cancer. Further studies are required to confirm these results.

ACKNOWLEDGMENTS

The authors would like to thank all of the patients and clinical investigators who were involved in the studies selected for this meta-analysis.

COMMENTS

Background

Although the prognosis of gastric cancer has improved, patients with gastric cancer still have a poor prognosis. The present prognostic factors do not fully predict individual clinical outcome. As a result, there is great interest in finding better markers to identify patients with a poor prognosis at the time of diagnosis.

Research frontiers

Meta-analysis was used to evaluate the prognostic role of hypoxia inducible factor-1α (HIF-1α) expression in gastric cancer in this study.

Innovations and breakthroughs

To the best knowledge, this is the first comprehensive and detailed meta-analysis to assess the association of HIF-1α expression with 5-year overall survival and tumor clinicopathological features in gastric cancer.

Applications

This study reported that the HIF-1α positive expression was associated with poor 5-year overall survival and clinicopathological features in patients with gastric cancer. In addition, HIF-1α positive expression could be a useful prognostic marker for gastric cancer.

Peer review

This manuscript describes an interesting meta-analysis of the HIF-1α positive expression associated with poor 5-year overall survival and clinicopathological features in patients with gastric cancer. In addition, HIF-1α positive expression could be a useful prognostic marker for gastric cancer. The manuscript was very well prepared and written and can be accepted for publication.

REFERENCES

1 **Dicken BJ**, Bigam DL, Cass C, Mackey JR, Joy AA, Hamilton SM. Gastric adenocarcinoma: review and considerations for future directions. *Ann Surg* 2005; **241**: 27-39 [PMID: 15621988 DOI: 10.1097/01.sla.0000149300.28588.23]

2 **Zhang H**, Sun LL, Meng YL, Song GY, Hu JJ, Lu P, Ji B. Survival trends in gastric cancer patients of Northeast China. *World J Gastroenterol* 2011; **17**: 3257-3262 [PMID: 21912476 DOI: 10.3748/wjg.v17.i27.3257]

3 **Liu JL**, Gao W, Kang QM, Zhang XJ, Yang SG. Prognostic value of survivin in patients with gastric cancer: a systematic review with meta-analysis. *PLoS One* 2013; **8**: e71930 [PMID: 23936532 DOI: 10.1371/journal.pone.0071930]

4 **Lim JY**, Yoon SO, Hong SW, Kim JW, Choi SH, Cho JY. Thioredoxin and thioredoxin-interacting protein as prognostic markers for gastric cancer recurrence. *World J Gastroenterol* 2012; **18**: 5581-5588 [PMID: 23112551 DOI: 10.3748/wjg.v18.i39.5581]

5 **Harris AL**. Hypoxia--a key regulatory factor in tumour growth. *Nat Rev Cancer* 2002; **2**: 38-47 [PMID: 11902584 DOI: 10.1038/nrc704]

6 **Zheng SS**, Chen XH, Yin X, Zhang BH. Prognostic significance of HIF-1α expression in hepatocellular carcinoma: a meta-analysis. *PLoS One* 2013; **8**: e65753 [PMID: 23799043 DOI: 10.1371/journal.pone.0065753]

7 **Marton I**, Knezevic F, Ramic S, Milosevic M, Tomas D. Immunohistochemical expression and prognostic significance of HIF-1α and VEGF-C in neuroendocrine breast cancer. *Anticancer Res* 2012; **32**: 5227-5232 [PMID: 23225420]

8 **Zhang ZG**, Zhang QN, Wang XH, Tian JH. Hypoxia-inducible factor 1 alpha (HIF-1α) as a prognostic indicator in patients with gastric tumors: a meta-analysis. *Asian Pac J Cancer Prev* 2013; **14**: 4195-4198 [PMID: 23991975]

9 **Zhan H**, Liang H, Liu X, Deng J, Wang B, Hao X. Expression of Rac1, HIF-1α, and VEGF in gastric carcinoma: correlation with angiogenesis and prognosis. *Onkologie* 2013; **36**: 102-107 [PMID: 23485997 DOI: 10.1159/000348525]

10 **Lu XX**, Chen YT, Feng B, Mao XB, Yu B, Chu XY. Expression and clinical significance of CD73 and hypoxia-inducible factor-1α in gastric carcinoma. *World J Gastroenterol* 2013; **19**: 1912-1918 [PMID: 23569336 DOI: 10.3748/wjg.v19.i12.1912]

11 **Isobe T**, Aoyagi K, Koufuji K, Shirouzu K, Kawahara A, Taira T, Kage M. Clinicopathological significance of hypoxia-inducible factor-1 alpha (HIF-1α) expression in gastric cancer. *Int J Clin Oncol* 2013; **18**: 293-304 [PMID: 22350022 DOI: 10.1007/s10147-012-0378-8]

12 **Qiu MZ**, Han B, Luo HY, Zhou ZW, Wang ZQ, Wang FH, Li YH, Xu RH. Expressions of hypoxia-inducible factor-1α and hexokinase-II in gastric adenocarcinoma: the impact on prognosis and correlation to clinicopathologic features. *Tumour Biol* 2011; **32**: 159-166 [PMID: 20845004 DOI: 10.1007/s13277-010-0109-6]

13 **Oh SY**, Kwon HC, Kim SH, Jang JS, Kim MC, Kim KH, Han JY, Kim CO, Kim SJ, Jeong JS, Kim HJ. Clinicopathologic significance of HIF-1alpha, p53, and VEGF expression and preoperative serum VEGF level in gastric cancer. *BMC Cancer* 2008; **8**: 123 [PMID: 18452596 DOI: 10.1186/1471-2407-8-123]

14 **Kolev Y**, Uetake H, Takagi Y, Sugihara K. Lactate dehydrogenase-5 (LDH-5) expression in human gastric cancer: association with hypoxia-inducible factor (HIF-1alpha) pathway, angiogenic factors production and poor prognosis. *Ann Surg Oncol* 2008; **15**: 2336-2344 [PMID: 18521687 DOI: 10.1245/s10434-008-9955-5]

15 **Cabuk D**, Basaran G, Celikel C, Dane F, Yumuk PF, Iyikesici MS, Ekenel M, Turhal NS. Vascular endothelial growth factor, hypoxia-inducible factor 1 alpha and CD34 expressions in early-stage gastric tumors: relationship with pathological factors and prognostic impact on survival. *Oncology* 2007; **72**: 111-117 [PMID: 18025805 DOI: 10.1159/000111118]

16 **Sumiyoshi Y**, Kakeji Y, Egashira A, Mizokami K, Orita H, Maehara Y. Overexpression of hypoxia-inducible factor 1alpha and p53 is a marker for an unfavorable prognosis in gastric cancer. *Clin Cancer Res* 2006; **12**: 5112-5117 [PMID:

16951228 DOI: 10.1158/1078-0432.CCR-05-2382]
17 **Mizokami K**, Kakeji Y, Oda S, Irie K, Yonemura T, Konishi F, Maehara Y. Clinicopathologic significance of hypoxia-inducible factor 1alpha overexpression in gastric carcinomas. *J Surg Oncol* 2006; **94**: 149-154 [PMID: 16847924 DOI: 10.1002/jso.20568]
18 **Lin S**, Chen ZH, Jiang HG, Yu JR. Robotic thyroidectomy versus endoscopic thyroidectomy: a meta-analysis. *World J Surg Oncol* 2012; **10**: 239 [PMID: 23140528 DOI: 10.1186/1477-7819-10-239]
19 **Lin S**, Jiang HG, Chen ZH, Zhou SY, Liu XS, Yu JR. Meta-analysis of robotic and laparoscopic surgery for treatment of rectal cancer. *World J Gastroenterol* 2011; **17**: 5214-5220 [PMID: 22215947 DOI: 10.3748/wjg.v17.i47.5214]
20 **Matsumoto K**, Arao T, Tanaka K, Kaneda H, Kudo K, Fujita Y, Tamura D, Aomatsu K, Tamura T, Yamada Y, Saijo N, Nishio K. mTOR signal and hypoxia-inducible factor-1 alpha regulate CD133 expression in cancer cells. *Cancer Res* 2009; **69**: 7160-7164 [PMID: 19738050 DOI: 10.1158/0008-5472.CAN-09-1289]
21 **Lin MT**, Kuo IH, Chang CC, Chu CY, Chen HY, Lin BR, Sureshbabu M, Shih HJ, Kuo ML. Involvement of hypoxia-inducing factor-1alpha-dependent plasminogen activator inhibitor-1 up-regulation in Cyr61/CCN1-induced gastric cancer cell invasion. *J Biol Chem* 2008; **283**: 15807-15815 [PMID: 18381294 DOI: 10.1074/jbc.M708933200]
22 **Xu LF**, Ni JY, Sun HL, Chen YT, Wu YD. Effects of hypoxia-inducible factor-1α silencing on the proliferation of CBRH-7919 hepatoma cells. *World J Gastroenterol* 2013; **19**: 1749-1759 [PMID: 23555163 DOI: 10.3748/wjg.v19.i11.1749]
23 **Li C**, Lu HJ, Na FF, Deng L, Xue JX, Wang JW, Wang YQ, Li QL, Lu Y. Prognostic role of hypoxic inducible factor expression in non-small cell lung cancer: a meta-analysis. *Asian Pac J Cancer Prev* 2013; **14**: 3607-3612 [PMID: 23886153]
24 **Yang Y**, Sun M, Wang L, Jiao B. HIFs, angiogenesis, and cancer. *J Cell Biochem* 2013; **114**: 967-974 [PMID: 23225225 DOI: 10.1002/jcb.24438]
25 **Bertout JA**, Patel SA, Simon MC. The impact of O2 availability on human cancer. *Nat Rev Cancer* 2008; **8**: 967-975 [PMID: 18987634 DOI: 10.1038/nrc2540]
26 **Abraham NS**, Byrne CJ, Young JM, Solomon MJ. Meta-analysis of well-designed nonrandomized comparative studies of surgical procedures is as good as randomized controlled trials. *J Clin Epidemiol* 2010; **63**: 238-245 [PMID: 19716267 DOI: 10.1016/j.jclinepi.2009.04.005]

P- Reviewers: Gurzu S, Munoz M, Yamakawa M
S- Editor: Gou SX **L- Editor**: Wang TQ **E- Editor**: Liu XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.1114

World J Gastroenterol 2014 January 28; 20(4): 1114-1118
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Family case of achalasia cardia: Case report and review of literature

Yulia Viktorovna Evsyutina, Alexander Sergeevich Trukhmanov, Vladimir Trophimovich Ivashkin

Yulia Viktorovna Evsyutina, Alexander Sergeevich Trukhmanov, Vladimir Trophimovich Ivashkin, Department of Internal Disease Propaedeutics, Gastroenterology and Hepatology, Sechenov First Moscow State Medical University, Moscow 119991, Russian Federation

Author contributions: Ivashkin VT designed the report; Evsyutina YuV was attending doctor for the patient; Trukhmanov AS performed pneumatic dilatation and performed image diagnosis; Ivashkin VT organized the report; and Evsyutina YuV wrote paper.

Correspondence to: Ivashkin Vladimir Trophimovich, PhD, Professor, Department of Internal Disease Propaedeutics, Gastroenterology and Hepatology, Sechenov First Moscow State Medical University, Pogodinskaya str., 1/1, Moscow 119991, Russian Federation. gastro@orc.ru
Telephone: +7-926-5288648 Fax: +7-499-2483610
Received: July 28, 2013 Revised: September 7, 2013
Accepted: September 16, 2013
Published online: January 28, 2014

Abstract

Achalasia cardia is an idiopathic disease that occurs as a result of inflammation and degeneration of myenteric plexi leading to the loss of postganglionic inhibitory neurons required for relaxation of the lower esophageal sphincter and peristalsis of the esophagus. The main symptoms of achalasia are dysphagia, regurgitation, chest pain and weight loss. At present, there are three main hypotheses regarding etiology of achalasia cardia which are under consideration, these are genetic, infectious and autoimmune. Genetic theory is one of the most widely discussed. Case report given below represents an inheritable case of achalasia cardia which was not diagnosed for a long time in an 81-year-old woman and her 58-year-old daughter.

Key words: Achalasia cardia; Dysphagia; Regurgitation; Gene polymorphism; Gastrostomy

Core tip: We report an inheritable case of achalasia cardia in an 81-year-old woman and her 58-year-old daughter with early manifestation of the disease at 23 and 25 years of age, respectively, and further progression of achalasia cardia which led to its decompensation and resulted in gastrostomy in the woman which was performed when she was 79-year-old.

Evsyutina YuV, Trukhmanov AS, Ivashkin VT. Family case of achalasia cardia: Case report and review of literature. *World J Gastroenterol* 2014; 20(4): 1114-1118 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/1114.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.1114

INTRODUCTION

Achalasia cardia is a primary esophageal motility disorder with involvement of the Auerbach's intermuscular plexus[1]. Achalasia cardia is considered a very rare disease, its incidence rate is 10 cases per 100000 population, and morbidity rate is 1 per 100000 population[2]. Achalasia cardia is diagnosed in adults most frequently in the age group of 25 to 60 years. Although the disease was first described by Thomas Williams, an English doctor, in 1674[3], the etiology of achalasia cardia still remains unknown. Genetic theory is one of the possible theories considered as the pathogenesis of achalasia cardia. We report an inheritable case of achalasia cardia which was not diagnosed for a long time in mother and her daughter.

CASE REPORT

Patient A, a 58-year-old, was admitted to the Clinic of the First Moscow State Medical University on March 2013 with the following complaints: dysphagia (to solids and liquids), chest pain during swallowing, food regurgitation,

Table 1 Complaints of the 58-year-old woman before treatment, in 2 mo after treatment, and the 79-year-old woman (modificated Eckardt score)

	Symptom	Score by frequency of symptoms			
		0	1	2	3
The 58-year-old woman before treatment	Dysphagia to solids	None	Occasionally	Daily	**Each meal**
	Dysphagia to liquids	None	Occasionally	Daily	**Each meal**
	Active regurgitation	None	Occasionally	**Daily**	Each meal
	Passive regurgitation	None	**Occasionally**	Daily	After each meal
	Spastic chest pain	None	**Occasionally**	Daily	Each meal
	Burning chest pain	**None**	Occasionally	Daily	After each meal
	Weight loss, kg	**None**	< 5	5-10	> 10
	Nocturnal cough	None	Monthly	Weekly	**Each night**
	Nocturnal dyspnea	**None**	Monthly	Weekly	Each night
	Hiccup	None	**Monthly**	Weekly	Daily
The 58-year-old woman in 2 mo after treatment	Dysphagia to solids	**None**	Occasionally	Daily	Each meal
	Dysphagia to liquids	**None**	Occasionally	Daily	Each meal
	Active regurgitation	**None**	Occasionally	Daily	Each meal
	Passive regurgitation	None	**Occasionally**	Daily	After each meal
	Spastic chest pain	None	**Occasionally**	Daily	Each meal
	Burning chest pain	**None**	Occasionally	Daily	After each meal
	Weightoss, kg	**None**	< 5	5-10	> 10
	Nocturnal cough	None	**Monthly**	Weekly	Each night
	Nocturnal dyspnea	**None**	Monthly	Weekly	Each night
	Hiccup	**None**	Monthly	Weekly	Daily
The 79-year-old woman	Dysphagia to solids	None	Occasionally	Daily	**Each meal**
	Dysphagia to liquids	None	Occasionally	Daily	**Each meal**
	Active regurgitation	None	Occasionally	Daily	**Each meal**
	Passive regurgitation	None	Occasionally	**Daily**	After each meal
	Spastic chest pain	None	Occasionally	Daily	**Each meal**
	Burning chest pain	None	**Occasionally**	Daily	After each meal
	Weight loss, kg	None	< 5	5-10	**> 10**
	Nocturnal cough	None	Monthly	Weekly	**Each night**
	Nocturnal dyspnea	None	Monthly	**Weekly**	Each night
	Hiccup	**None**	Monthly	Weekly	Daily

and nocturnal cough.

Medical history of this case shows that the patient considered herself ill since she was 23 years of age, when she noticed for 1st time symptoms of dysphagia which disturbed her at least once in 2 mo, and these symptoms continued until she was 31-year-old when her episodes of dysphagia became more frequent and occurred once or twice per week. The patient underwent contrast-enhanced X-ray examination of the esophagus (which revealed: stricture of cardiac portion of the esophagus up to 1.5 cm, suprastenotic dilatation of the esophagus up to 4 cm, delayed evacuation of barium meal from the esophagus to the stomach and absence of gastric air bubble). Achalasia cardia was diagnosed. The patient refused to the proposed therapy, as her sense of physical well-being before 2003 was satisfactory, until she developed new complications of pressing pain behind her sternum during meals, regurgitation after meals and nocturnal cough. Since 2012 the patient noted that swallowing of foods (both solids and liquids were difficult at every meal (she reported that the first swallow was difficult but the further swallows were normal) (Table 1).

General performance status was rather satisfactory at the time of admission to the hospital. Body mass index was 30.4 kg/m^2 (class I obesity). Skin and visible mucosa were normal. Breathing was harsh above the lungs; no abnormal breath sounds were heard. Heart sounds were rhythmic and muffled. Heart rate was 70 beats per minute. Arterial blood pressure was 130/70 mmHg. Palpation revealed that abdomen was soft and painless in all areas. Liver could be palpated at the edge of the right costal arch. Costovertebral angle tenderness was negative at both sides.

Diagnostic findings: complete blood count - hemoglobin 138 g/L, erythrocytes 4.3 × 10^{12}, leukocytes 4.5 × 10^9, platelets 269.2 × 10^9, ESR 5 mm/h. Blood biochemistry: total protein 8.0 g/dL, albumin 4.2 g/dL, creatinine 1.0 mg/dL. Clinical urine analysis showed that all findings were within normal range. Esophagogastroduodenoscopy showed that the patient's esophagus had very elastic walls and the esophageal lumen was enlarged up to 4 cm. There were foamy mucus in the lumen, esophageal mucosa was hyperemic in the lower third and had grayish-pearl color tone. The cardiac region was closed. Moderate amount of bile was found in the stomach, folds of mucosa were high and longitudinally-wavy. Stomach mucosa was thin and was hyperemic in the antrum region. Contrast-enhanced X-ray examination of the esophagus: barium meal test revealed that the act of swallowing was not impaired, fluid level was determined during the fasting state in Th8 projection. Width of the esophagus was 4 cm and the outlet of the esophagus was 0.8 cm (Figure 1A). Tertiary contractions of esophageal wall were observed (Figure 1B). The esophagus periodi-

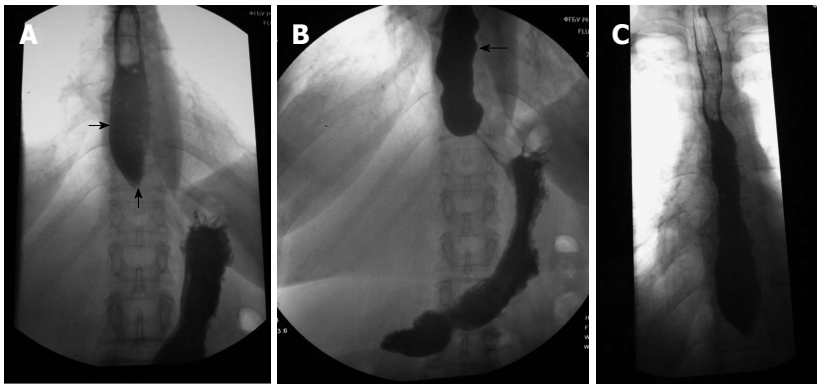


Figure 1 A timed barium esophagogram. A: Dilatation of esophagus (arrow), beak-like narrowing of LES (arrow); B: The third contractions of esophagus (arrow); C: In 20 min after swallowing of barium, almost two thirds of the barium can be seen in the esophagus.

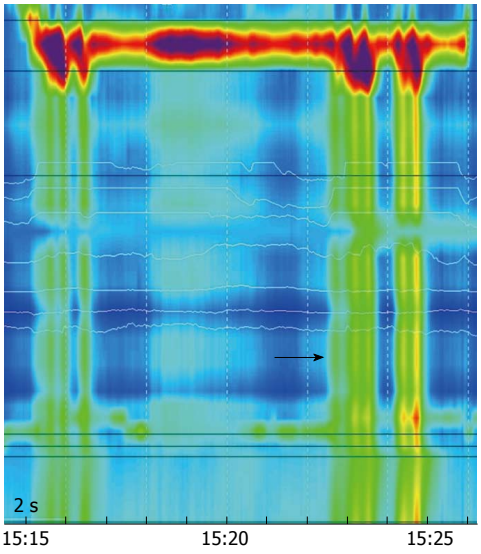


Figure 2 A high resolution manometry. Type Ⅱ achalasia (Chicago classification): Resting Pressure lower esophageal sphincter (LES) 37 mmHg, IRP4, Integrated Relaxation Pressure 20 mmHg, Lack of normal esophageal peristalsis in response to normal swallowing of water (arrow).

cally emptied with small portions. 2/3 of contrast materials were detected in the esophagus 20 minutes after the start of examination (Figure 1C).

According to the findings of high resolution manometry, the following deserved high attention: Resting Pressure of lower esophageal sphincter was 37 mmHg, Integrated Relaxation Pressure was 20 mmHg and there was absence of normal peristaltic contractions of the esophagus in response to wet swallows (Figure 2).

Course of the treatment with spasmolytics and antacids was performed in the clinic as the first stage of treatment. In view of Ⅱ type of achalasia cardia, 3 sessions of pneumatic dilatation of the cardia were performed at the second stage of treatment using balloon with a diameter of 3.5 cm. Pressure was built up to 140-230 mmHg, procedure lasted for about 60 s. The patient underwent the procedure satisfactorily without any complications. The patient's state of physical well-being improved after dilatation of the cardia: dysphagia after ingestion of solids and liquids was resolved, and there were no passive and active regurgitation, no chest pain and episodes of nocturnal cough reduced to once per week.

Two months after the above conducted treatment, the patient's complaints were assessed again (Table 1) and the sum of scores reduced from 14 to 3 which indicated the success of the conducted treatment.

The patient's mother, (Patient P) an 81-year-old, also suffers from achalasia cardia. History of her disease: patient was diagnosed with a congenital elongation of the esophagus during her childhood. Since the age of 25 she noted dysphagia after ingestion of solid food which occurred very rarely, at least once or twice per month. Since the age of 55, the patient noted episodes of chest pain upon swallowing. Since the age of 78, the patient noted a significant worsening in her state of physical well-being which included difficulty in swallowing at every meal, and also vomiting of food which had just been ingested. The patient underwent X-ray examination of the esophagus which revealed that there was a marked dilatation of the lower third of the esophagus up to 10 cm, rough deformation with multiple cascade folds, and she was diagnosed with achalasia cardia. However the patient was not proposed to undergo treatment regarding her old age and her state of physical well-being continued to worsen with occurrence of dysphagia after ingestion of liquid food in addition to the above mentioned complaints. The patient lost almost 15 kg during 1 year (Table 1). And she was urgently hospitalized in inpatient surgical department where she was examined and esophagogastroduodenoscopy findings revealed: stricture of the lower third of the esophagus up to 1/3 of the lumen, atrophic gastritis with hemorrhagic component. X-ray examination revealed significant dilatation of the esophagus up to 11 cm and rough deformations with multiple cascade folds (Figure 3A and B).

Patient underwent surgery in 2011 which involved the placement of a stent through constricted esophagus in the stomach, however it proved to be ineffective as the patient still had vomiting of just ingested food and impaired movement of liquid food through the esophagus during the postoperative period, So within a week gastrostomy was performed on the patient and the patient received nutrition through the G-tube.

DISCUSSION

This case report represents vertical inheritance of acha-

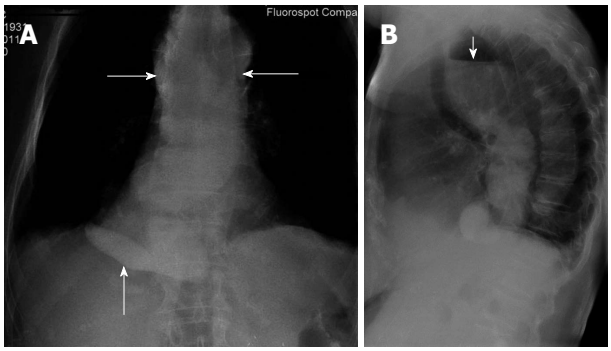


Figure 3 A timed barium esophagogram. A: A very severe dilatation of esophagus up to 11 cm and a coarse deformation of esophagus with lots of folds in the form of cascade (arrow); B: Lateral side, a severe dilatation and deformation of esophagus. The level of liquid in esophagus can be seen (arrow).

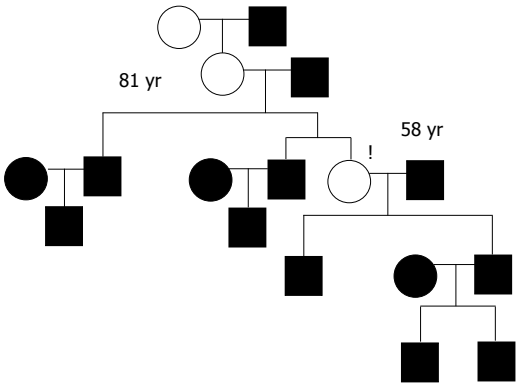


Figure 4 A pedigree of the family. Occurrence of achalasia with a vertical line of transmission (esophageal achalasia in 58-year-old daughter, 81-year-old mother and grandmother).

lasia cardia (Figure 4). An interview revealed that our patient's grandmother had suffered for 20 years from dysphagia until her death at the age of 60 due to stroke and no examination of esophagus was performed on her.

It is to be noted that this disease manifested with dysphagia at a rather early age of 23-25 both in the woman and her daughter and presented with entire clinical symptoms like dysphagia, passive and active regurgitation, chest pain which developed just after many years. Also it is necessary to notice that both the patients had nocturnal coughs, and the woman had nocturnal dyspnea which indicated the state of decompensation of achalasia cardia and absolute need for therapy.

Method of treatment for achalasia cardia of the patient's mother is of great interest, especially the placement of stent through constricted esophagus in the stomach that is usually used as a palliative method of treatment of esophageal tumors. This explains unfavourable postoperative period with persisted dysphagia and vomiting as the placed stent prevents the lower esophageal sphincter from opening. In view of long-lasting disease and rough deformation of the esophagus the patient was subjected to gastrostomy to allow nutrition, at present she still lives with gastrostome. This surgery severely affected patient's social life and caused a persistent inflammation of skin around the gastrostome.

As of genetic theory, first of all genetic syndromes seen in pediatric practice and associated with the development of achalasia cardia should be mentioned. Mutation of ALADIN 12q13 gene is the most common cause of achalasia cardia in children, it leads to the development of autosomal-recessive disease, so called All grove syndrome or AAA syndrome which is characterized by the development of achalasia, alacrimia and Addison's disease[4].

Risk of achalasia is also increased in children with Down's syndrome. Approximately 75% of children with trisomy 21 have gastrointestinal diseases and 2% develop achalasia[5]. Risk of achalasia in children with Down's syndrome is 200 time higher than in normal population[6]. Besides Down's syndrome, incidence of achalasia cardia is significantly higher in children with Rozycki syndrome and Pierre-Robin syndrome.

Speaking about the adult population, polymorphism of some genes is by all means important in the development of achalasia cardia. It is proved that by the theory of polymorphism of IL23R gene localized on chromosome Ip31. It is also supported by the study on IL23R Arg381 Gln gene polymorphism in 262 patient with achalasia and 802 healthy volunteers which was done in Spain. Results have also revealed that this gene polymorphism had occurred in men who had suffered from achalasia (less than 40 years of age), which allows us to conclude that IL23R is very important as a predisposing factor in the development of idiopathic achalasia cardia[7].

IL10 promoter haplotype GCC was associated with the development of idiopathic achalasia cardia in patients of the same Spanish population[8].

In addition, a link was found between achalasia cardia and specific HLA-genotype. Study conducted in 2002 had investigated the level of circulating autoantibodies and HLA DQA1 and DQB1 alleles in patients with achalasia and healthy volunteers and demonstrated that autoantibodies to Auerbach's plexus were revealed in all women and 66.7% of men with idiopathic achalasia and DQA1 × 0103 and DQB1 × 0603-alleles[9].

It's also important to note the theory of polymorphism of NO-synthase (NOS) which is a fragment catalyzing the production of nitrogen oxide from arginine, oxygen and NADPH. There are 3 different types of NOS: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). Their responsible genes were located on chromosomes: 12q24.2, 17q11.2-q12 and 7q36. Some works have reported polymorphism of all 3 genes in patients with achalasia. Of these, the polymorphism of iNOS22 × A/Ab and eNOS × 4a4a were those which were most frequently detected[10,11].

Besides nitrogen oxide, vasoactive intestinal peptide is the second neurotransmitter of inhibitory neurons. One of its receptors, Receptor 1 which belongs to the secretin family, is expressed by immune cells such as T- lymphocytes, macrophages and dendritic cells[12]. Polymorphism of this gene (*VIPR1*) can also play an important role in the development of idiopathic achalasia. *VIPR1* gene is

localized on chromosome 3p22 and some studies have reported five simple nucleotide polymorphisms of this gene such as (rs421558) *Intron-1*, (rs437876) *Intron-4*, (rs417387) *Intron-6*, rs896 and rs9677 (*3'UTR*)[13].

Genes responsible for the synthesis of protein tyrosine phosphatase, nonreceptor type 22 (*PTPN22*) are localized in chromosome 1p13.м3-p13 and is associated with the development of autoimmune diseases[14]. Lymphoid-specific phosphatase (Lyp), one of the phosphatases that are coded by this gene, is an intracellular tyrosine phosphatase which is an important regulator of T-cell activation[15]. C1858T polymorphism of *PTPN22* gene (when codon 620 Arg (R) is replaced by Trp (W) resulting in production of Lyp-W620 instead of Lyp-R620 that leads to an increase in T-lymphocyte activity) is an important risk factor for the development of autoimmune disease[16,17]. The study conducted in Spain also revealed that the polymorphism described above increased the risk of achalasia in Spanish population[18].

In conclusion it should be noted that this case report illustrates the genetic theory of development of achalasia cardia. Genetic analysis which is currently widely performed in patients with achalasia helped a lot to answer to the question regarding etiology of this disease, however there is need for more intensive study in this field.

REFERENCES

1 **Park W**, Vaezi MF. Etiology and pathogenesis of achalasia: the current understanding. *Am J Gastroenterol* 2005; **100**: 1404-1414 [PMID: 15929777 DOI: 10.1111/j.1572-0241.2005.41775.x]

2 **Spechler SJ**. Clinical manifestations and diagnosis of achalasia. Uptodate. [Internet] 2010 [cited 2011 April 19]. Available from: URL: http: //www.uptodate.com/contents/clinical-manifestations-and-diagnosis-ofachalasia

3 **Willis T**. Pharmaceutice ratioalis sive diatribe de medicamentarum operationibus in humano corpore. London: Hagia Comitis, 1674

4 **Tullio-Pelet A**, Salomon R, Hadj-Rabia S, Mugnier C, de Laet MH, Chaouachi B, Bakiri F, Brottier P, Cattolico L, Penet C, Bégeot M, Naville D, Nicolino M, Chaussain JL, Weissenbach J, Munnich A, Lyonnet S. Mutant WD-repeat protein in triple-A syndrome. *Nat Genet* 2000; **26**: 332-335 [PMID: 11062474 DOI: 10.1038/81642]

5 **Moore SW**. Down syndrome and the enteric nervous system. *Pediatr Surg Int* 2008; **24**: 873-883 [PMID: 18633623 DOI: 10.1007/s00383-008-2188-7]

6 **Zárate N**, Mearin F, Gil-Vernet JM, Camarasa F, Malagelada JR. Achalasia and Down's syndrome: coincidental association or something else? *Am J Gastroenterol* 1999; **94**: 1674-1677 [PMID: 10364043 DOI: 10.1016/S0002-9270(99)00217-8]

7 **Emami MH**, Raisi M, Amini J, Daghaghzadeh H. Achalasia and thyroid disease. *World J Gastroenterol* 2007; **13**: 594-599 [PMID: 17278227]

8 **Nuñez C**, García-González MA, Santiago JL, Benito MS, Mearín F, de la Concha EG, de la Serna JP, de León AR, Urcelay E, Vigo AG. Association of IL10 promoter polymorphisms with idiopathic achalasia. *Hum Immunol* 2011; **72**: 749-752 [PMID: 21641950 DOI: 10.1016/j.humimm.2011.05.017]

9 **Ruiz-de-León A**, Mendoza J, Sevilla-Mantilla C, Fernández AM, Pérez-de-la-Serna J, Gónzalez VA, Rey E, Figueredo A, Díaz-Rubio M, De-la-Concha EG. Myenteric antiplexus antibodies and class II HLA in achalasia. *Dig Dis Sci* 2002; **47**: 15-19 [PMID: 11837716 DOI: 10.1023/A:1013242831900]

10 **Vigo AG**, Martínez A, de la Concha EG, Urcelay E, Ruiz de León A. Suggested association of NOS2A polymorphism in idiopathic achalasia: no evidence in a large case-control study. *Am J Gastroenterol* 2009; **104**: 1326-1327 [PMID: 19337240 DOI: 10.1038/ajg.2009.72]

11 **Mearin F**, García-González MA, Strunk M, Zárate N, Malagelada JR, Lanas A. Association between achalasia and nitric oxide synthase gene polymorphisms. *Am J Gastroenterol* 2006; **101**: 1979-1984 [PMID: 16848803 DOI: 10.1111/j.1572-0241.2006.00762.x]

12 **Paladini F**, Cocco E, Cauli A, Cascino I, Vacca A, Belfiore F, Fiorillo MT, Mathieu A, Sorrentino R. A functional polymorphism of the vasoactive intestinal peptide receptor 1 gene correlates with the presence of HLA-B*2705 in Sardinia. *Genes Immun* 2008; **9**: 659-667 [PMID: 18668120 DOI: 10.1038/gene.2008.60]

13 **Paladini F**, Cocco E, Cascino I, Belfiore F, Badiali D, Piretta L, Alghisi F, Anzini F, Fiorillo MT, Corazziari E, Sorrentino R. Age-dependent association of idiopathic achalasia with vasoactive intestinal peptide receptor 1 gene. *Neurogastroenterol Motil* 2009; **21**: 597-602 [PMID: 19309439 DOI: 10.1111/j.1365-2982.2009.01284.x]

14 **de León AR**, de la Serna JP, Santiago JL, Sevilla C, Fernández-Arquero M, de la Concha EG, Nuñez C, Urcelay E, Vigo AG. Association between idiopathic achalasia and IL23R gene. *Neurogastroenterol Motil* 2010; **22**: 734-738, e218 [PMID: 20367798 DOI: 10.1111/j.1365-2982.2010.01497.x]

15 **Jawaheer D**, Seldin MF, Amos CI, Chen WV, Shigeta R, Etzel C, Damle A, Xiao X, Chen D, Lum RF, Monteiro J, Kern M, Criswell LA, Albani S, Nelson JL, Clegg DO, Pope R, Schroeder HW, Bridges SL, Pisetsky DS, Ward R, Kastner DL, Wilder RL, Pincus T, Callahan LF, Flemming D, Wener MH, Gregersen PK. Screening the genome for rheumatoid arthritis susceptibility genes: a replication study and combined analysis of 512 multicase families. *Arthritis Rheum* 2003; **48**: 906-916 [PMID: 12687532 DOI: 10.1002/art.10989]

16 **van Oene M**, Wintle RF, Liu X, Yazdanpanah M, Gu X, Newman B, Kwan A, Johnson B, Owen J, Greer W, Mosher D, Maksymowych W, Keystone E, Rubin LA, Amos CI, Siminovitch KA. Association of the lymphoid tyrosine phosphatase R620W variant with rheumatoid arthritis, but not Crohn's disease, in Canadian populations. *Arthritis Rheum* 2005; **52**: 1993-1998 [PMID: 15986374 DOI: 10.1002/art.21123]

17 **Ghoshal UC**, Daschakraborty SB, Singh R. Pathogenesis of achalasia cardia. *World J Gastroenterol* 2012; **18**: 3050-3057 [PMID: 22791940 DOI: 10.3748/wjg.v18.i24.3050]

18 **Santiago JL**, Martínez A, Benito MS, Ruiz de León A, Mendoza JL, Fernández-Arquero M, Figueredo MA, de la Concha EG, Urcelay E. Gender-specific association of the PTPN22 C1858T polymorphism with achalasia. *Hum Immunol* 2007; **68**: 867-870 [PMID: 17961776 DOI: 10.1016/j.humimm.2007.07.005]

P- Reviewers: Jani K, Schuchert MJ **S- Editor**: Qi Y
L- Editor: A **E- Editor**: Wang CH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.1119

World J Gastroenterol 2014 January 28; 20(4): 1119-1122
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Endoscopic transgastric drainage of a gastric wall abscess after endoscopic submucosal dissection

Osamu Dohi, Moyu Dohi, Ken Inoue, Yasuyuki Gen, Masayasu Jo, Kazuhiko Tokita

Osamu Dohi, Moyu Dohi, Ken Inoue, Yasuyuki Gen, Masayasu Jo, Kazuhiko Tokita, Department of Gastroenterology and Hepatology, North Medical Center, Kyoto Prefectural University of Medicine, Kyoto 629-2261, Japan
Author contributions: Dohi O wrote the manuscript; Dohi M, Inoue K, Gen Y, Jo M and Tokita K were also involved in editing the critical revision of the manuscript for important intellectual content.
Correspondence to: Osamu Dohi, MD, PhD, Department of Gastroenterology and Hepatology, North Medical Center, Kyoto Prefectural University of Medicine, 481 Otokoyama Yosano-Cho, Yosa-gun, Kyoto 629-2261, Japan. osamu-d@koto.kpu-m.ac.jp
Telephone: +81-772-463371 Fax: +81-772-463371
Received: April 23, 2013 Revised: July 31, 2013
Accepted: August 16, 2013
Published online: January 28, 2014

Abstract

A 63-year-old woman was referred to our hospital for further examination because of an incidental finding of early gastric cancer. Endoscopic submucosal dissection (ESD) was successfully performed for complete resection of the tumor. On the first post-ESD day, the patient suddenly complained of abdominal pain after an episode of vomiting. Abdominal computed tomography (CT) showed delayed perforation after ESD. The patient was conservatively treated with an intravenous proton pump inhibitor and antibiotics. On the fifth post-ESD day, CT revealed a gastric wall abscess in the gastric body. Gastroscopy revealed a gastric fistula at the edge of the post-ESD ulcer, and pus was found flowing into the stomach. An intradrainage stent and an extradrainage nasocystic catheter were successfully inserted into the abscess for endoscopic transgastric drainage. After the procedure, the clinical symptoms and laboratory test results improved quickly. Two months later, a follow-up CT scan showed no collection of pus. Consequently, the intradrainage stent was removed. Although the gastric wall abscess recurred 2 wk after stent removal, it recovered soon after endoscopic transgastric drainage. Finally, after stent removal and oral antibiotic treatment for 1 mo, no recurrence of the gastric wall abscess was found.

Key words: Gastric wall abscess; Transgastric drainage; Delayed perforation; Endoscopic submucosal dissection; Early gastric cancer

Core tip: In this report, we describe for the first time a case in which a gastric wall abscess caused by delayed perforation after endoscopic submucosal dissection was conservatively treated with endoscopic drainage *via* the gastric lumen and antibiotics.

Dohi O, Dohi M, Inoue K, Gen Y, Jo M, Tokita K. Endoscopic transgastric drainage of a gastric wall abscess after endoscopic submucosal dissection. *World J Gastroenterol* 2014; 20(4): 1119-1122 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/1119.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.1119

INTRODUCTION

Endoscopic submucosal dissection (ESD), a technique developed in Japan, is associated with a high success rate for en-bloc resection in early gastric cancer (EGC). This procedure is associated with a low risk of lymph node metastasis, but a high risk of such complications as perforation. The risk of perforation during ESD is approximately 4%[1]. In contrast, delayed perforation is a rare complication, that occurs 1 to 2 d after the procedure and often requires emergency surgery[2,3]. One study reported that such perforations occurred in 6 (0.5%) out of 1159 consecutive patients with 1329 EGCs who underwent ESD[2]. Another report indicated two cases (0.43%) of

delayed perforation occurring after the completion of ESD for 468 gastric noninvasive gastric neoplasias including EGCs and gastric adenomas[3]. In a few cases, however, delayed perforations were treated with conservative therapy instead of surgery[4-6]. A gastric wall abscess is an uncommon suppurative infection of the stomach. The prognosis of gastric wall abscess has recently improved because of the use of endoscopic drainage techniques and antibiotics[7-10]. Here, we report a case in which a patient developed a gastric wall abscess after delayed perforation caused by ESD for EGC. Surgery was avoided in this case, and the patient was conservatively treated with endoscopic transgastric drainage and antibiotics.

CASE REPORT

A 63-year-old woman with suspected EGC on the gastric body based on gastroscopy, was referred to our hospital for further examination. The patient's medical history and laboratory test results were unremarkable. A 65-mm-wide I+IIa lesion was localized in the greater curvature of the lower part of the gastric body (Figure 1A). Examination of biopsy specimens confirmed a well-differentiated adenocarcinoma. No abnormalities were detected upon abdominal computed tomography (CT). The lesion was safely and completely removed en-bloc with ESD (Figure 1B), using an endoscope (GIF-Q260J, Olympus, Tokyo, Japan), an electrosurgical generator (VIO 300D, Erbe Co., Tübingen, Germany), a needle-knife (KD-10Q-1, Olympus, Tokyo, Japan), and an insulated-tip (IT) knife 2 (KD-611 L, Olympus, Tokyo, Japan). The size of the mucosa that was resected en-bloc was 75 mm × 60 mm (length × width), including 65 mm × 40 mm of the cancer lesion (Figure 1C). On the first post-ESD day, the patient suddenly complained of abdominal pain after an episode of vomiting. Initial enhanced abdominal CT showed a portion of the thickened gastric wall and a small amount of free air and ascites around the stomach (Figure 2A). The patient was diagnosed with delayed perforation after ESD. Because the CT revealed a small amount of free air and ascites, we expected the delayed perforation to be very small, and we did not perform emergency endoscopy. The patient was treated with a regimen that comprised fasting, an intravenous proton pump inhibitor, and the antibiotic cefotiam (CTM 3g/d). On the fifth post-ESD day, despite the continued absence of abdominal pain, the patient was febrile. Abdominal T2-weighted magnetic resonance imaging (MRI) showed a moderately high-intensity tumor with niveau formation, 5 cm in size, located in the posterior wall of the fundus of the stomach (Figure 2B). The images demonstrated a gastric wall abscess in the gastric body. Gastroscopic reexamination revealed a smooth, elevated lesion shaped like a submucosal tumor at the greater curvature of the upper body on the oral side of the post-ESD ulcer (Figure 3A). A gastric fistula was found at the edge of the post-ESD ulcer, and pus was found to flow into the stomach (Figure 3B). An endoscopic retrograde cholangiopancreatography (ERCP) catheter (MTW Endoskopie, Wesel, Germany) was inserted through the fistula into the abscess. Then, two 0.035-inch guidewires (VisiGlide; Olympus, Tokyo, Japan) were inserted over the catheter into the abscess. Finally, a 7.0-Fr double pigtail stent (Zimmon Biliary Stent, Cook Japan, Tokyo, Japan) for the internal fistula and a 7.5-Fr nasocystic catheter (Flexima ENBD Catheter, Boston Scientific Japan, Tokyo, Japan) for the external fistula were successfully inserted into the abscess using the double-wire technique for endoscopic transgastric drainage (Figure 3C). Five days later, the abscess contents had cleared, the nasocystic catheter was removed, and the stent was left in to allow continued drainage. A week after the procedure, the patient was released from the hospital. At discharge,

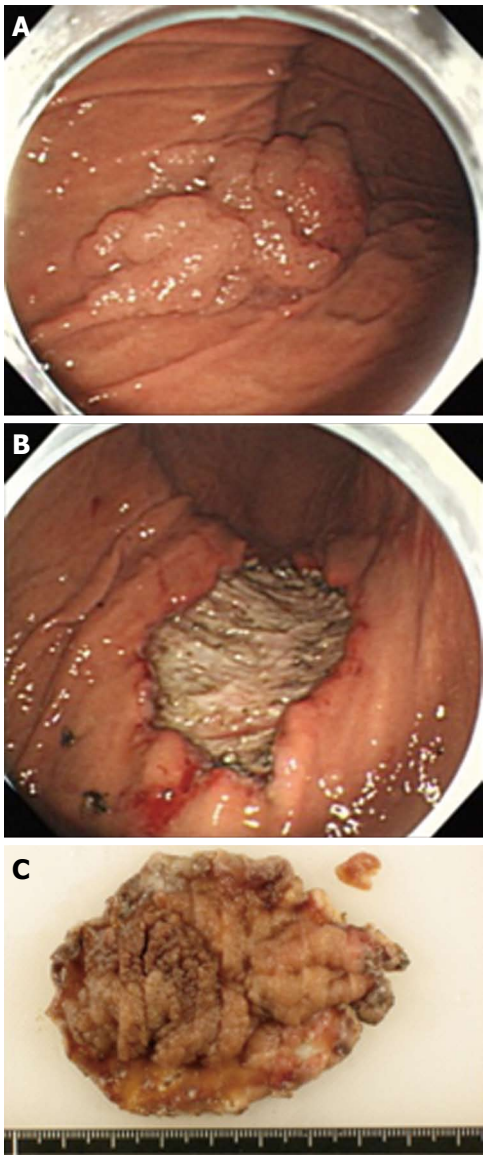


Figure 1 Endoscopic submucosal dissection for early gastric cancer. A: A cancerous lesion, an early gastric cancer, macroscopic type 0-I+IIa, 75 mm in diameter, is observed on the greater curvature of the gastric body; B: The lesion was successfully removed en bloc without gastric perforation; C: The lesion with the surrounding mucosa was cut into 2-mm-wide serial-step sections.

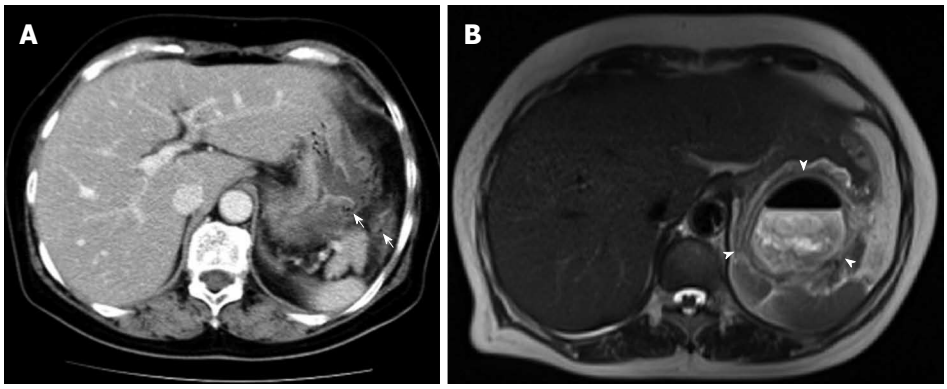


Figure 2 Abdominal computed tomography and magnetic resonance imaging findings. A: Abdominal computed tomography (CT) revealed a portion of the thickened gastric wall and a small amount of ascites and free air around the stomach. Free air is indicated by the arrow; B: Abdominal T2-weighted magnetic resonance imaging showed a moderately high-intensity tumor with niveau formation, 5 cm in size, located in the posterior wall of the fundus of the stomach, which suggested focal abscess formation. The abscess is indicated by the arrowhead.

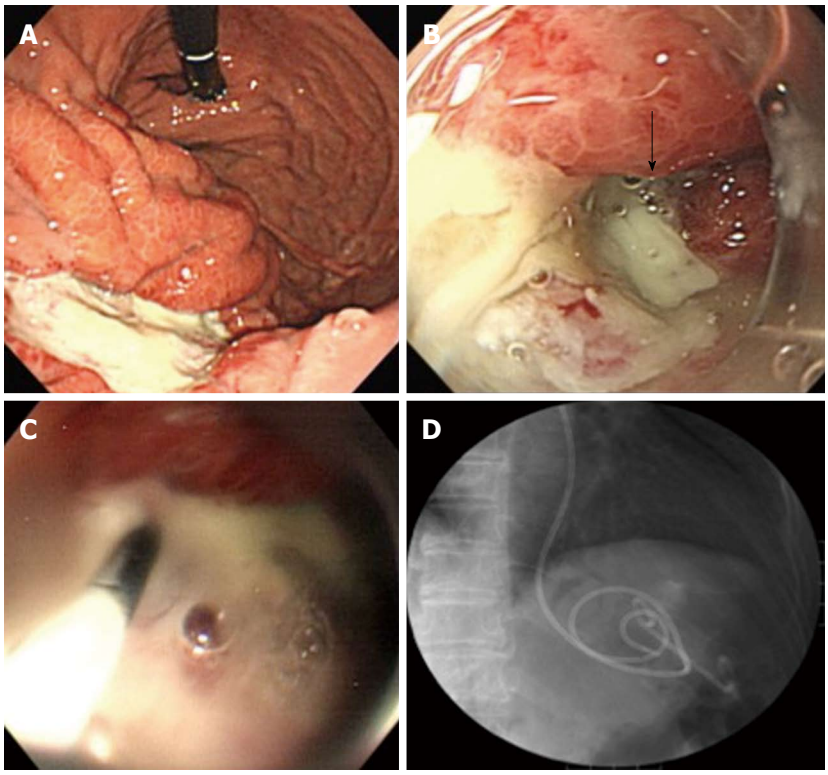


Figure 3 Endoscopic transgastric drainage for a gastric wall abscess. A: Gastroscopy revealed a smooth, elevated lesion at the greater curvature of the upper body on the oral side of the post-endoscopic submucosal dissection ulcer; B: A gastric fistula was found on the edge of the post-endoscopic submucosal dissection ulcer. The fistula is indicated by an arrow; C: An endoscopic retrograde cholangiopancreatography-catheter was inserted through the fistula into the abscess; D: A plastic stent placement for the internal fistula and a nasocystic catheter for the external fistula were successfully inserted into the abscess.

she was not prescribed any antibiotics and was scheduled to be followed up at our hospital. When a follow-up CT scan conducted 2 mo later showed the absence of pus collection, the intradrainage stent was removed. Two weeks after the intradrainage stent removal, the patient developed fever and epigastralgia. CT revealed a recurrence of the gastric wall abscess in the gastric body. Gastroscopy revealed a gastric fistula in the center of the post-ESD ulcer scar. As before, an ERCP catheter (MTW Endoskopie, Wesel, Germany) was inserted through the fistula into the abscess. Endoscopic transgastric drainage was repeated by inserting the intradrainage stent and the extradrainage catheter into the abscess. A few days later, the abscess contents had cleared, and the nasocystic catheter was removed. Two months later, a follow-up CT scan showed no collection of pus. The intradrainage stent was then removed, and an oral antibiotic (amoxicillin 750 mg/d) was administered for 1 mo. A follow-up CT scan conducted 1 mo later revealed no abnormal findings. At the 24-mo follow-up examination, the patient was asymptomatic, and the abdominal CT scan showed no abnormalities.

DISCUSSION

The mechanism underlying delayed perforation is thought to involve electrical cautery during submucosal dissection or repeated coagulation that causes ischemic changes in the gastric wall and results in necrosis[3]. The shape of the delayed perforation was round, and the color of the surrounding muscle layer was whitish, suggesting necrosis of the muscle layer related to the delayed perforation[6]. In our case, necrosis of the muscle layer by electrical cautery combined with an increase in the abdominal pressure caused by vomiting may have led to the perforation because its location was coincident with the lesion coagulated with electrical cautery; moreover, the patient had suddenly experienced peritoneal irritation after vomiting. Emergency surgery was not required in our case because the peritonitis was localized and improved with conservative antibiotics treatment.

Until recently, surgical drainage using antibiotics was the recommended treatment for a gastric wall abscess. However, surgical therapy has been replaced by technically advanced radiologic and endoscopic interventions. Recent studies have reported that the prognosis of gastric wall abscess has improved because of the introduction of endoscopic drainage with or without antibiotics[7-11]. To our knowledge, this is the first report of a case in which a gastric wall abscess caused by delayed perforation after ESD was treated with endoscopic drainage *via* the gastric lumen and antibiotics. Because there was a fistula between the abscess and the post-ESD ulcer, endoscopic transgastric drainage was easily performed. A pigtail catheter was left in the stomach for 2 mo to allow additional drainage of the purulent fluid. Although the ESD ulcer was almost healed, the gastric wall abscess recurred because of a residual fistula after stent removal. Therefore, it was necessary to continue antibiotic treatment until the fistula closed. Although delayed perforations after ESD can be conservatively treated, endoscopists must keep in mind that a gastric wall abscess may develop and that it is necessary to perform the procedure carefully.

REFERENCES

1 **Gotoda T**, Yamamoto H, Soetikno RM. Endoscopic submucosal dissection of early gastric cancer. *J Gastroenterol* 2006; **41**: 929-942 [PMID: 17096062 DOI: 10.1007/s00535-006-1954-3]

2 **Hanaoka N**, Uedo N, Ishihara R, Higashino K, Takeuchi Y, Inoue T, Chatani R, Hanafusa M, Tsujii Y, Kanzaki H, Kawada N, Iishi H, Tatsuta M, Tomita Y, Miyashiro I, Yano M. Clinical features and outcomes of delayed perforation after endoscopic submucosal dissection for early gastric cancer. *Endoscopy* 2010; **42**: 1112-1115 [PMID: 21120780 DOI: 10.1055/s-0030-1255932]

3 **Kato M**, Nishida T, Tsutsui S, Komori M, Michida T, Yamamoto K, Kawai N, Kitamura S, Zushi S, Nishihara A, Nakanishi F, Kinoshita K, Yamada T, Iijima H, Tsujii M, Hayashi N. Endoscopic submucosal dissection as a treatment for gastric noninvasive neoplasia: a multicenter study by Osaka University ESD Study Group. *J Gastroenterol* 2011; **46**: 325-331 [PMID: 21107615 DOI: 10.1007/s00535-010-0350-1]

4 **Onozato Y**, Iizuka H, Sagawa. A case report of delayed perforation due to endoscopic submucosal dissection (ESD) for early gastric cancer. *Progr Dig Endosc* 2006; **68**: 114-115

5 **Hirasawa T**, Yamamoto Y, Okada K. A case of the delayed perforation due to endoscopic submucosal dissection for the gastric cancer of the residual stomach. *Progr Dig Endosc* 2009; **74**: 52-53

6 **Ikezawa K**, Michida T, Iwahashi K, Maeda K, Naito M, Ito T, Katayama K. Delayed perforation occurring after endoscopic submucosal dissection for early gastric cancer. *Gastric Cancer* 2012; **15**: 111-114 [PMID: 21948482 DOI: 10.1007/s10120-011-0089-2]

7 **Choong NW**, Levy MJ, Rajan E, Kolars JC. Intramural gastric abscess: case history and review. *Gastrointest Endosc* 2003; **58**: 627-629 [PMID: 14560756]

8 **Will U**, Masri R, Bosseckert H, Knopke A, Schönlebe J, Justus J. Gastric wall abscess, a rare endosonographic differential diagnosis of intramural tumors: successful endoscopic treatment. *Endoscopy* 1998; **30**: 432-435 [PMID: 9689523 DOI: 10.1055/s-2007-1001297]

9 **Eley A**, Richards HG. A case of localized gastric abscess. *Br J Surg* 1959; **47**: 97-98 [PMID: 13819886 DOI: 10.1002/bjs.18004720119]

10 **Marcos WC**, Petrini BG, Xavier RL, Starling RM, Couto JC, Ribeiro GJ. Gastric wall abscess--an uncommon condition treated by an alternative form. *Clinics (Sao Paulo)* 2010; **65**: 819-821 [PMID: 20835563 DOI: 10.1590/S1807-59322010000800015]

11 **Yang CW**, Yen HH. Endoscopic sonography in the diagnosis and treatment of a gastric wall abscess: a case report and review of the literature. *J Clin Ultrasound* 2012; **40**: 105-108 [PMID: 22228085 DOI: 10.1002/jcu.20780]

P- Reviewers: Grassi R, Shi RH, Yoshi N **S- Editor**: Wen LL
L- Editor: A **E- Editor**: Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i4.1123

World J Gastroenterol 2014 January 28; 20(4): 1123-1126
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Laparoscopic partial cystectomy with mucosal stripping of extraluminal duodenal duplication cysts

Jeik Byun, Hyoung-Min Oh, Soo-Hong Kim, Hyun-Young Kim, Sung-Eun Jung, Kwi-Won Park, Woo-Sun Kim

Jeik Byun, Hyoung-Min Oh, Soo-Hong Kim, Hyun-Young Kim, Sung-Eun Jung, Kwi-Won Park, Department of Pediatric Surgery, Seoul National University Children's Hospital, Seoul 110-744, South Korea
Woo-Sun Kim, Department of Radiology, Seoul National University Hospital, Seoul 110-744, South Korea
Author contributions: Byun J, Oh HM and Kim HY designed the report; Oh HM, Kim SH, Kim HY and Park KW were attending doctors for the patient; Kim HY performed the operation; Kim WS performed diagnostic imaging; Jung SE organized the report; Byun J wrote the paper.
Correspondence to: Hyun-Young Kim, MD, Department of Pediatric Surgery, Seoul National University Children's Hospital, 101 Daehang-ro, Yeongeon-dong, Jongro-gu, Seoul 110-744, South Korea. spkhy02@snu.ac.kr
Telephone: +82-2-20722478 Fax: +82-2-7475130
Received: September 2, 2013 Revised: October 12, 2013
Accepted: November 12, 2013
Published online: January 28, 2014

Abstract

Duodenal duplication cysts are rare congenital anomalies. Duodenal duplication should be considered in the differential diagnosis of patients who present with abdominal symptoms with cystic structures neighboring the duodenum. Here, we present an 8-year-old girl with a duodenal duplication cyst treated with partial cystectomy with mucosal stripping performed laparoscopically. Laparoscopic surgery can be considered as a treatment option for duodenal duplication cysts, especially in extraluminal locations.

Key words: Duodenal duplication cyst; Laparoscopic surgery; Partial cystectomy; Extraluminal situation; Children

Core tip: In duodenal duplication cysts, the endoscopic approach has limitations in extraluminal situations. Endoscopic internal derivation cannot remove the mucosal layer where malignancy mainly occurs, therefore, we propose that laparoscopic surgery can be considered as a treatment option for duodenal duplication cysts, especially in extraluminal locations.

Byun J, Oh HM, Kim SH, Kim HY, Jung SE, Park KW, Kim WS. Laparoscopic partial cystectomy with mucosal stripping of extraluminal duodenal duplication cysts. *World J Gastroenterol* 2014; 20(4): 1123-1126 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i4/1123.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i4.1123

INTRODUCTION

Duodenal duplication cysts are rare congenital anomalies, which can appear during the neonatal period or later in life depending on the degree of the gastric outlet obstruction[1]. Classical treatment for duodenal duplication cysts is total resection, but in cases requiring pancreaticoduodenectomy, less-invasive approaches have been proposed[2-4]. Here, we describe the laparoscopic technique for partial resection of duodenal duplication cysts in an 8-year-old girl.

CASE REPORT

An 8-year-old girl had suffered from intermittent abdominal pain, nausea and vomiting for 2 mo. The patient had no other underlying diseases. The abdomen was flat and no definite mass was palpable. The laboratory studies were normal. The patient did not have jaundice and had normal serum bilirubin level. The tumor markers were not checked before surgery. Abdominal ultrasonography (US) showed 6 cm × 5 cm lobulated retroperitoneal cystic mass, septated between the duodenum

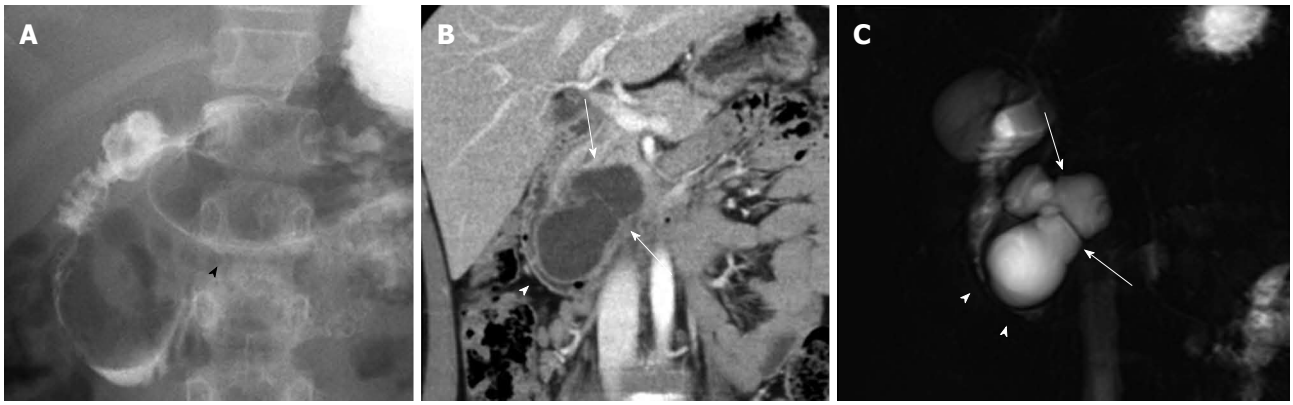


Figure 1 Images for preoperative diagnosis. A: Upper gastrointestinal series demonstrated luminal narrowing and displacement of the duodenum by the mass (arrow head); B, C: Coronal reconstruction computed tomography image and coronal thick-slab T2-weighted image showed a multiloculated cystic mass (arrows) between the head of the pancreas and duodenum. The mass seemed to originate from the uncinate process of the pancreas, and the second and third portions of the duodenum were inferolaterally displaced and compressed by the mass (arrow heads).

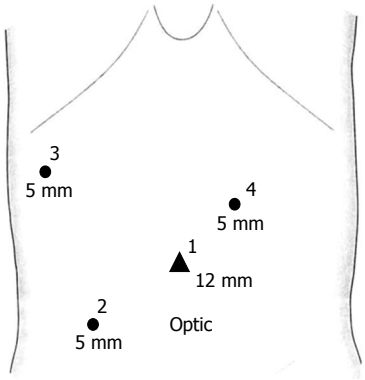


Figure 2 Laparoscopic port insertion site. 1: 12-mm optical umbilical port; 2, 5-mm main working port; 3: 5-mm port for liver retraction.

and the pancreas. Upper gastrointestinal series revealed luminal narrowing of the second portion of the duodenum (Figure 1A). Abdominal computed tomography (CT) and biliary pancreas magnetic resonance imaging (MRI) showed a multiseptated cystic mass suspected of originating from the uncinate process of the pancreas. Extra compression from this lesion seemed to be causing narrowing of the duodenal lumen (Figure 1B and C). Retroperitoneal lymphangioma of the pancreas was primarily suspected, along with other differential diagnoses including solid and papillary epithelial neoplasms, pancreatoblastoma, unusual cystadenoma, and pancreatic pseudocyst.

Laparoscopic exploration was performed. The patient was placed in the supine position under general anesthesia and an optical umbilical port was placed under direct vision followed by three additional ports (Figure 2). A Snowden-Pencer Snake Retractor (CareFusion, San Diego, CA, United States) was inserted through port site 3 for liver retraction. After performing a laparoscopic Kocher maneuver, a multiloculated cystic mass was identified in the second portion of the duodenum. The cystic mass originated from the mesenteric border of the duodenum and adhered to the uncinate process of the pancreas (Figure 3A). After adhesiolysis between the cyst and the pancreas, clear demarcation of the cystic surface was identified (Figure 3B). An arterial branch supplying the mass originating from the gastroduodenal artery was ligated with a 5-mm hemoclip and divided (Figure 3C). The proximal border of the mass was easily dissected from duodenum with an ENDOPATH Electrosurgery Probe Plus II System with a Hook electrode (Ethicon Endo-Surgery, Cincinnati, OH, United States), but the distal border was directly attached to the duodenal wall, forming a common wall. A harmonic scalpel (Ethicon Endo-Surgery) was used to resect the mass from the duodenum and the remnant mucosa was cauterized with the ENDOPATH system. The lesion formed a common wall with the duodenum, without communication or fistula. No intraoperative complications were encountered.

The patient was discharged on Postoperative Day 9 without any complications. Upon histopathological review, a compatible duodenal wall with partially denuded epithelium was consistent with duodenal duplication (Figure 4).

DISCUSSION

Patients with duodenal duplication cysts present with recurrent nausea, vomiting, abdominal mass, abdominal distension, pancreatitis, and gastrointestinal bleeding[4,5]. Duodenal duplication can be diagnosed with various imaging modalities. The "double-layered wall" of the duodenum seen with US, CT, and endoscopic ultrasound (EUS) are used to reach a diagnosis of duodenal duplication[6-8]. In the present case, the patient had complained of recurrent nausea and vomiting. However, in the US findings, the double-layered wall was not significant, and the lesion seemed to originate from the pancreas in the CT and MR images. Moreover, the lesion showed multiseptations, which turned out to be the folding patterns of the cyst wall upon surgical exploration, and made it

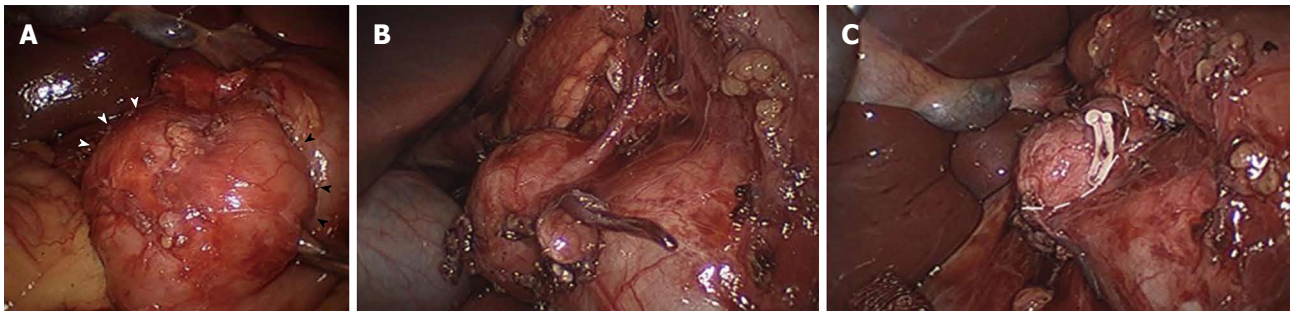


Figure 3 Laparoscopic procedures and intraoperative findings. A: Laparoscopic intraoperative findings of duodenal duplication cyst after Kocher's maneuver. Duplication cyst (white arrow heads) and mesenteric side of posterior wall of duodenum (black arrow heads); B: Demarcation of the mass surface after adhesiolysis. An arterial branch from the gastroduodenal artery supporting the mass was also noticed; C: Resection line with harmonic scalpel (white line).

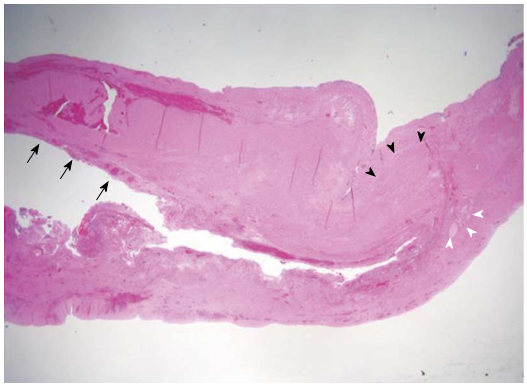


Figure 4 Pathological findings of resected specimen showing duodenal wall with partially denuded epithelium (hematoxylin and eosin staining, ×12.5). The mucosal lining (black arrows), smooth muscle coat (black arrowheads), and glands (white arrowheads) were noticed.

difficult to distinguish from other pancreatic tumors, including pancreatic lymphangioma.

The ideal treatment for duodenal duplication cysts is complete surgical resection if their location allows it without endangering the biliopancreatic ducts[4]. However, in cases of duodenal duplication cysts involving important nearby structures, for example, the pancreas or biliary ducts, major surgical procedures like pancreaticoduodenectomy may be required for total resection. This major procedure has a high complication rate resulting in poor quality of life especially in children, therefore, less-invasive approaches, for instance, partial resection or internal marsupialization, have been proposed[2-4]. We have performed partial cystectomy with mucosal stripping without duodenotomy using laparoscopic devices. However, in children, the small abdominal cavity and relatively small organs are limitations to laparoscopic approaches. Compared with conventional open surgery, laparoscopic surgery is less invasive and has more cosmetic advantages if it is performed by an experienced surgeon with the proper equipment.

Endoscopic therapy for duodenal duplication has been suggested recently for minimally invasive treatment. However, the endoscopic approach has limitations for extraluminal cysts. Endoscopic internal derivation cannot remove the mucosal layer where malignancy mainly occurs, therefore, we propose that laparoscopic surgery is a safer method, especially for cases with extraluminal locations[2,9,10].

In summary, although duodenal duplication cysts are rare, they should be considered in the differential diagnosis of patients who present with abdominal symptoms with cystic structures neighboring the duodenum. Laparoscopic partial cystectomy with mucosal stripping can be considered as a treatment option for duodenal duplication cysts even in children.

REFERENCES

1 **Schalamon J**, Schleef J, Höllwarth ME. Experience with gastro-intestinal duplications in childhood. *Langenbecks Arch Surg* 2000; **385**: 402-405 [PMID: 11127525]
2 **Seeliger B**, Piardi T, Marzano E, Mutter D, Marescaux J, Pessaux P. Duodenal duplication cyst: a potentially malignant disease. *Ann Surg Oncol* 2012; **19**: 3753-3754 [PMID: 22832999 DOI: 10.1245/s10434-012-2502-4]
3 **Siddiqui AM**, Shamberger RC, Filler RM, Perez-Atayde AR, Lillehei CW. Enteric duplications of the pancreatic head: definitive management by local resection. *J Pediatr Surg* 1998; **33**: 1117-1120; discussion 1117-1120 [PMID: 9694106 DOI: 10.1016/S0022-3468(98)90543-7]
4 **Merrot T**, Anastasescu R, Pankevych T, Tercier S, Garcia S, Alessandrini P, Guys JM. Duodenal duplications. Clinical characteristics, embryological hypotheses, histological findings, treatment. *Eur J Pediatr Surg* 2006; **16**: 18-23 [PMID: 16544221 DOI: 10.1055/s-2006-923798]
5 **Chen JJ**, Lee HC, Yeung CY, Chan WT, Jiang CB, Sheu JC. Meta-analysis: the clinical features of the duodenal duplication cyst. *J Pediatr Surg* 2010; **45**: 1598-1606 [PMID: 20713206 DOI: 10.1016/j.jpedsurg.2010.01.010]
6 **Barr LL**, Hayden CK, Stansberry SD, Swischuk LE. Enteric duplication cysts in children: are their ultrasonographic wall characteristics diagnostic? *Pediatr Radiol* 1990; **20**: 326-328 [PMID: 2190153 DOI: 10.1007/BF02013165]
7 **Antaki F**, Tringali A, Deprez P, Kwan V, Costamagna G, Le Moine O, Delhaye M, Cremer M, Devière J. A case series of symptomatic intraluminal duodenal duplication cysts: presentation, endoscopic therapy, and long-term outcome (with video). *Gastrointest Endosc* 2008; **67**: 163-168 [PMID: 18155438 DOI: 10.1016/j.gie.2007.08.006]
8 **Okuyama H**, Matsuo Y, Fukui Y, Imura K, Kamata S, Okada A. Intrapancreatic duodenal duplication associated with pancreatic pseudocysts. *J Pediatr Surg* 1992; **27**: 1573-1574

[PMID: 1469579 DOI: 10.1016/0022-3468(92)90512-6]
9 **Jiménez M**, Cadière GB, Dapri G, Vasilikostas G, Bruyns J, Capelluto E. Duodenal duplication cyst in an adult: first simultaneous laparoscopic and endoscopic surgery. *J Laparoendosc Adv Surg Tech A* 2009; **19**: 207-210 [PMID: 19361290 DOI: 10.1089/lap.2008.0015]
10 **Vandenbroucke F**, Dagenais M, Létourneau R, Lapointe R, Roy A. Endoscopic treatment of a duodenal duplication cyst. *Endoscopy* 2005; **37**: 601 [PMID: 15933941 DOI: 10.1055/s-2005-861328]

P- Reviewers: Chiu CC, Shamim D **S- Editor**: Zhai HH
L- Editor: Kerr C **E- Editor**: Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2014 January 28; 20(4): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

GENERAL INFORMATION

World Journal of Gastroenterology (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access (OA) journal. *WJG* was established on October 1, 1995. It is published weekly on the 7th, 14th, 21st, and 28th each month. The *WJG* Editorial Board consists of 1339 experts in gastroenterology and hepatology from 67 countries.

Aims and scope

The primary task of *WJG* is to rapidly publish high-quality original articles, reviews, and commentaries in the fields of gastroenterology, hepatology, gastrointestinal endoscopy, gastrointestinal surgery, hepatobiliary surgery, gastrointestinal oncology, gastrointestinal radiation oncology, gastrointestinal imaging, gastrointestinal interventional therapy, gastrointestinal infectious diseases, gastrointestinal pharmacology, gastrointestinal pathophysiology, gastrointestinal pathology, evidence-based medicine in gastroenterology, pancreatology, gastrointestinal laboratory medicine, gastrointestinal molecular biology, gastrointestinal immunology, gastrointestinal microbiology, gastrointestinal genetics, gastrointestinal translational medicine, gastrointestinal diagnostics, and gastrointestinal therapeutics. *WJG* is dedicated to become an influential and prestigious journal in gastroenterology and hepatology, to promote the development of above disciplines, and to improve the diagnostic and therapeutic skill and expertise of clinicians.

WJG is published by Baishideng Publishing Group (BPG) in both electronic and online forms. All *WJG* articles are published in *WJG* website and PubMed Central. The major advantages of OA journals are faster release and delivery, no page or graph restrictions, and increased visibility, usage and impact. Full-text PDF articles and electronic/online versions are freely available to global readers. After the paper is published, the author(s) can obtain high-quality PDF files, which contain the journal cover, a list of editorial board members, table of contents, text, and back cover of the journal. BPG has a strong professional editorial team composed of editorial board members, editors-in-chief, science editors, language editors, and electronic editors. BPG currently publishes 43 OA clinical medical journals, including 42 in English, has a total of 15471 editorial board members or peer reviewers, and is a world first-class publisher.

Columns

The columns in the issues of *WJG* will include: (1) Editorial: The editorial board members are invited to make comments on an important topic in their field in terms of its current research status and future directions to lead the development of this discipline; (2) Frontier: The editorial board members are invited to select a highly cited cutting-edge original paper of his/her own to summarize major findings, the problems that have been resolved and remain to be resolved, and future research directions to help readers understand his/her important academic point of view and future research directions in the field; (3) Diagnostic Advances: The editorial board members are invited to write high-quality diagnostic advances in their field to improve the diagnostic skills of readers. The topic covers general clinical diagnosis, differential diagnosis, pathological diagnosis, laboratory diagnosis, imaging diagnosis, endoscopic diagnosis, biotechnological diagnosis, functional diagnosis, and physical diagnosis; (4) Therapeutics Advances: The editorial board members are invited to write high-quality therapeutic advances in their field to help improve the therapeutic skills of readers. The topic covers medication therapy, psychotherapy, physical therapy, replacement therapy, interventional therapy, minimally invasive therapy, endoscopic therapy, transplantation therapy, and surgical therapy; (5) Field of Vision: The editorial board members are invited to write commentaries on classic articles, hot topic articles, or latest articles to keep readers at the forefront of research and increase their levels of clinical research. Classic articles refer to papers that are included in Web of Knowledge and have received a large number of citations (ranking in the top 1%) after being published for more than years, reflecting the quality and impact of papers. Hot topic articles refer to papers that are included in Web of Knowledge and have received a large number of citations after being published for no more than 2 years, reflecting cutting-edge trends in scientific research. Latest articles refer to the latest published high-quality papers that are included in PubMed, reflecting the latest research trends. These commentary articles should focus on the status quo of research, the most important research topics, the problems that have now been resolved and remain to be resolved, and future research directions. Basic information about the article to be commented (including authors, article title, journal name, year, volume, and inclusive page numbers; (6) Minireviews: The editorial board members are invited to write short reviews on recent advances and trends in research of molecular biology, genomics, and related cutting-edge technologies to provide readers with the latest knowledge and help improve their diagnostic and therapeutic skills; (7) Review: To make a systematic review to focus on the status quo of research, the most important research topics, the problems that have now been resolved and remain to be resolved, and future research directions; (8) Topic Highlight: The editorial board members are invited to write a series of articles (7-10 articles) to comment and discuss a hot topic to help improve the diagnostic and therapeutic skills of readers; (9) Medical Ethics: The editorial board members are invited to write articles about medical ethics to increase readers' knowledge of medical ethics. The topic covers international ethics guidelines, animal studies, clinical trials, organ transplantation, *etc.*; (10) Clinical Case Conference or Clinicopathological Conference: The editorial board members are invited to contribute high-quality clinical case conference; (11) Original Articles: To report innovative and original findings in gastroenterology and hepatology; (12) Brief Articles: To briefly report the novel

and innovative findings in gastroenterology and hepatology; (13) Meta-Analysis: Covers the systematic review, mixed treatment comparison, meta-regression, and overview of reviews, in order to summarize a given quantitative effect, *e.g.*, the clinical effectiveness and safety of clinical treatments by combining data from two or more randomized controlled trials, thereby providing more precise and externally valid estimates than those which would stem from each individual dataset if analyzed separately from the others; (14) Case Report: To report a rare or typical case; (15) Letters to the Editor: To discuss and make reply to the contributions published in *WJG*, or to introduce and comment on a controversial issue of general interest; (16) Book Reviews: To introduce and comment on quality monographs of gastroenterology and hepatology; and (17) Autobiography: The editorial board members are invited to write their autobiography to provide readers with stories of success or failure in their scientific research career. The topic covers their basic personal information and information about when they started doing research work, where and how they did research work, what they have achieved, and their lessons from success or failure.

Name of journal
World Journal of Gastroenterology

ISSN
ISSN 1007-9327 (print)
ISSN 2219-2840 (online)

Launch date
October 1, 1995

Frequency
Weekly

Editorial office
Jin-Lei Wang, Director
Xiu-Xia Song, Vice Director
World Journal of Gastroenterology
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-59080039
Fax: +86-10-85381893
E-mail: bpgoffice@wjgnet.com
http://www.wjgnet.com

Publisher
Baishideng Publishing Group Co., Limited
Flat C, 23/F., Lucky Plaza,
315-321 Lockhart Road,
Wan Chai, Hong Kong, China
Fax: +852-65557188
Telephone: +852-31779906
E-mail: bpgoffice@wjgnet.com
http://www.wjgnet.com

Production center
Beijing Baishideng BioMed Scientific Co., Limited
Room 903, Building D, Ocean International Center,
No. 62 Dongsihuan Zhonglu, Chaoyang District,
Beijing 100025, China
Telephone: +86-10-85381892
Fax: +86-10-85381893

Representative office
USA Office
8226 Regency Drive,
Pleasanton, CA 94588-3144, United States

Instructions to authors
Full instructions are available online at http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm

Indexed and abstracted in
Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. ISI, Thomson Reuters, 2011 Impact Factor: 2.471 (32/74 Gastroenterology and Hepatology).

SPECIAL STATEMENT

All articles published in this journal represent the viewpoints of the authors except where indicated otherwise.

Biostatistical editing
Statistical review is performed after peer review. We invite an expert in Biomedical Statistics to evaluate the statistical method used in the paper, including *t* test (group or paired comparisons), chi-squared test, ridit, probit, logit, regression (linear, curvilinear, or stepwise), correlation, analysis of variance, analysis of covariance, *etc.* The reviewing points include: (1) Statistical methods should be described when they are used to verify the results; (2) Whether the statistical techniques are suitable or correct; (3) Only homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (*n*). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word "significantly" should be replaced by its synonyms (if it indicates extent) or the *P* value (if it indicates statistical significance).

Conflict-of-interest statement
In the interests of transparency and to help reviewers assess any potential bias, *WJG* requires authors of all papers to declare any competing commercial, personal, political, intellectual, or religious interests in relation to the submitted work.

Referees are also asked to indicate any potential conflict they might have reviewing a particular paper. Before submitting, authors are suggested to read "Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Ethical Considerations in the Conduct and Reporting of Research: Conflicts of Interest" from International Committee of Medical Journal Editors (ICMJE), which is available at: http://www.icmje.org/ethical_4conflicts.html.

Statement of informed consent

Manuscripts should contain a statement to the effect that all human studies have been reviewed by the appropriate ethics committee or it should be stated clearly in the text that all persons gave their informed consent prior to their inclusion in the study. Details that might disclose the identity of the subjects under study should be omitted. Authors should also draw attention to the Code of Ethics of the World Medical Association (Declaration of Helsinki, 1964, as revised in 2004).

Sample wording: [Name of individual] has received fees for serving as a speaker, a consultant and an advisory board member for [names of organizations], and has received research funding from [names of organization]. [Name of individual] is an employee of [name of organization]. [Name of individual] owns stocks and shares in [name of organization]. [Name of individual] owns patent [patent identification and brief description].

Statement of human and animal rights

When reporting the results from experiments, authors should follow the highest standards and the trial should conform to Good Clinical Practice (for example, US Food and Drug Administration Good Clinical Practice in FDA-Regulated Clinical Trials; UK Medicines Research Council Guidelines for Good Clinical Practice in Clinical Trials) and/or the World Medical Association Declaration of Helsinki. Generally, we suggest authors follow the lead investigator's national standard. If doubt exists whether the research was conducted in accordance with the above standards, the authors must explain the rationale for their approach and demonstrate that the institutional review body explicitly approved the doubtful aspects of the study.

Before submitting, authors should make their study approved by the relevant research ethics committee or institutional review board. If human participants were involved, manuscripts must be accompanied by a statement that the experiments were undertaken with the understanding and appropriate informed consent of each. Any personal item or information will not be published without explicit consents from the involved patients. If experimental animals were used, the materials and methods (experimental procedures) section must clearly indicate that appropriate measures were taken to minimize pain or discomfort, and details of animal care should be provided.

SUBMISSION OF MANUSCRIPTS

Manuscripts should be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of Baishideng Publishing Group Co., Limited, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the ICMJE to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is http://www.clinicaltrials.gov sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

Authors should retain one copy of the text, tables, photographs and illustrations because rejected manuscripts will not be returned to the author(s) and the editors will not be responsible for loss or damage to photographs and illustrations sustained during mailing.

Online submissions

Manuscripts should be submitted through the Online Submission System at: http://www.wjgnet.com/esps/. Authors are highly recommended to consult the ONLINE INSTRUCTIONS TO AUTHORS (http://www.wjgnet.com/1007-9327/g_info_20100315215714.htm) before attempting to submit online. For assistance, authors encountering problems with the Online Submission System may send an email describing the problem to bpgoffice@wjgnet.com, or by telephone: +86-10-5908-0039. If you submit your manuscript online, do not make a postal contribution. Repeated online submission for the same manuscript is strictly prohibited.

MANUSCRIPT PREPARATION

All contributions should be written in English. All articles must be submitted using word-processing software. All submissions must be typed in 1.5 line spacing and 12 pt. Book Antiqua with ample margins. Style should conform to our house format. Required information for each of the manuscript sections is as follows:

Title page

Title: Title should be less than 12 words.

Running title: A short running title of less than 6 words should be provided.

Authorship: Authorship credit should be in accordance with the standard proposed by ICMJE, based on (1) substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be published. Authors should meet conditions 1, 2, and 3.

Institution: Author names should be given first, then the complete name of institution, city, province and postcode. For example, Xu-Chen Zhang, Li-Xin Mei, Department of Pathology, Chengde Medical College, Chengde 067000, Hebei Province, China. One author may be represented from two institutions, for example, George Sgourakis, Department of General, Visceral, and Transplantation Surgery, Essen 45122, Germany; George Sgourakis, 2nd Surgical Department, Korgialenio-Benakio Red Cross Hospital, Athens 15451, Greece.

Author contributions: The format of this section should be:

Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed the research; Wang CL, Zou CC, Hong F and Wu XM performed the research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed the data; and Wang CL, Liang L and Fu JF wrote the paper.

Supportive foundations: The complete name and number of supportive foundations should be provided, e.g. Supported by National Natural Science Foundation of China, No. 30224801

Correspondence to: Only one corresponding address should be provided. Author names should be given first, then author title, affiliation, the complete name of institution, city, postcode, province, country, and email. All the letters in the email should be in lower case. A space interval should be inserted between country name and email address. For example, Montgomery Bissell, MD, Professor of Medicine, Chief, Liver Center, Gastroenterology Division, University of California, Box 0538, San Francisco, CA 94143, United States. montgomery.bissell@ucsf.edu

Telephone and fax: Telephone and fax should consist of +, country number, district number and telephone or fax number, *e.g.*, Telephone: +86-10-59080039 Fax: +86-10-85381893

Peer reviewers: All articles received are subject to peer review. Normally, three experts are invited for each article. Decision on acceptance is made only when at least two experts recommend publication of an article. All peer-reviewers are acknowledged on Express Submission and Peer-review System website.

Abstract

There are unstructured abstracts (no less than 200 words) and structured abstracts. The specific requirements for structured abstracts are as follows:

An informative, structured abstract should accompany each manuscript. Abstracts of original contributions should be structured into the following sections: AIM (no more than 20 words; Only the purpose of the study should be included. Please write the Aim in the form of "To investigate/study/…"), METHODS (no less than 140 words for Original Articles; and no less than 80 words for Brief Articles), RESULTS (no less than 150 words for Original Articles and no less than 120 words for Brief Articles; You should present *P* values where appropriate and must provide relevant data to illustrate how they were obtained, *e.g.*, 6.92 ± 3.86 *vs* 3.61 ± 1.67, $P < 0.001$), and CONCLUSION (no more than 26 words).

Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

Core tip

Please write a summary of less than 100 words to outline the most innovative and important arguments and core contents in your paper to attract readers.

Text

For articles of these sections, original articles and brief articles, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the main text or in Figures and Tables, but not in both.

Illustrations

Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A:...; B:...; C:...; D:...; E:...; F:...; G: …*etc.* It is our principle to publish high resolution-figures for the E-versions.

Tables

Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement, but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

Notes in tables and illustrations

Data that are not statistically significant should not be noted. $^{a}P < 0.05$, $^{b}P < 0.01$ should be noted ($P > 0.05$ should not be noted). If there are other series of *P* values, $^{c}P < 0.05$ and $^{d}P < 0.01$ are used. A third series of *P* values can be expressed as $^{e}P < 0.05$ and $^{f}P < 0.01$. Other notes in tables or under illustrations should be expressed as ^{1}F, ^{2}F, ^{3}F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscript and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

REFERENCES

Coding system

The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability[1,2]". If references are cited directly in the text, they should be put together within the text, for example, "From references[19,22-24], we know that...".

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

PMID and DOI

Pleased provide PubMed citation numbers to the reference list, *e.g.*, PMID and DOI, which can be found at http://www.ncbi.nlm.nihgov/sites/entrez?db=pubmed and http://www.crossref.

org/SimpleTextQuery/, respectively. The numbers will be used in E-version of this journal.

Style for journal references

Authors: the name of the first author should be typed in bold-faced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

Style for book references

Authors: the name of the first author should be typed in bold-faced letters. The surname of all authors should be typed with the initial letter capitalized, followed by their abbreviated middle and first initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR) Book title. Publication number. Publication place: Publication press, Year: start page and end page.

Format

Journals

English journal article (list all authors and include the PMID where applicable)

1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

Units

Use SI units. For example: body mass, m (B) = 78 kg; blood pressure, p (B) = 16.2/12.3 kPa; incubation time, t (incubation) = 96 h, blood glucose concentration, c (glucose) 6.4 ± 2.1 mmol/L; blood CEA mass concentration, p (CEA) = 8.6 24.5 μg/L; CO_2 volume fraction, 50 mL/L CO_2, not 5% CO_2; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, *etc.* Arabic numerals such as 23, 243, 641 should be read 23243641.

The format for how to accurately write common units and quantums can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315223018.htm.

Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.
Genotypes: *gyrA*, *arg* 1, *c myc*, *c fos*, *etc.*
Restriction enzymes: *Eco*RI, *Hin*dI, *Bam*HI, *Kbo* I, *Kpn* I, *etc.*
Biology: *H. pylori*, *E coli*, *etc.*

Examples for paper writing

All types of articles' writing style and requirement will be found in the link: http://www.wjgnet.com/esps/NavigationInfo.aspx?id=15.

RESUBMISSION OF THE REVISED MANUSCRIPTS

Authors must revise their manuscript carefully according to the revision policies of Baishideng Publishing Group Co., Limited. The revised version, along with the signed copyright transfer agreement, responses to the reviewers, and English language Grade A certificate (for non-native speakers of English), should be submitted to the online system *via* the link contained in the e-mail sent by the editor. If you have any questions about the revision, please send e-mail to esps@wjgnet.com.

Language evaluation

The language of a manuscript will be graded before it is sent for revision. (1) Grade A: priority publishing; (2) Grade B: minor language polishing; (3) Grade C: a great deal of language polishing needed; and (4) Grade D: rejected. Revised articles should reach Grade A.

Copyright assignment form

Please download a Copyright assignment form from http://www.wjgnet.com/1007-9327/g_info_20100315222818.htm.

Responses to reviewers

Please revise your article according to the comments/suggestions provided by the reviewers. The format for responses to the reviewers' comments can be found at: http://www.wjgnet.com/1007-9327/g_info_20100315222607.htm

Proof of financial support

For papers supported by a foundation, authors should provide a copy of the approval document and serial number of the foundation.

STATEMENT ABOUT ANONYMOUS PUBLICATION OF THE PEER REVIEWERS' COMMENTS

In order to increase the quality of peer review, push authors to carefully revise their manuscripts based on the peer reviewers' comments, and promote academic interactions among peer reviewers, authors and readers, we decide to anonymously publish the reviewers' comments and author's responses at the same time the manuscript is published online.

PUBLICATION FEE

WJG is an international, peer-reviewed, open access, online journal. Articles published by this journal are distributed under the terms of the Creative Commons Attribution Non-commercial License, which permits use, distribution, and reproduction in any medium, provided the original work is properly cited, the use is non commercial and is otherwise in compliance with the license. Authors of accepted articles must pay a publication fee. Publication fee: 1365 USD per article. All invited articles are published free of charge.

百世登
Baishideng®

Published by **Baishideng Publishing Group Co., Limited**
Flat C, 23/F., Lucky Plaza,
315-321 Lockhart Road, Wan Chai, Hong Kong, China
Fax: +852-65557188
Telephone: +852-31779906
E-mail: bpgoffice@wjgnet.com
http://www.wjgnet.com

ISSN 1007-9327