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

World Journal of *Gastroenterology*

World J Gastroenterol 2013 November 21; 19(43): 7489-7824

Baishideng Publishing Group
www.wjgnet.com

WJG World Journal of Gastroenterology

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

Editorial Board

2010-2013

The *World Journal of Gastroenterology* Editorial Board consists of 1352 members, representing a team of worldwide experts in gastroenterology and hepatology. They are from 64 countries, including Albania (1), Argentina (8), Australia (33), Austria (15), Belgium (14), Brazil (13), Brunei Darussalam (1), Bulgaria (2), Canada (21), Chile (3), China (82), Colombia (1), Croatia (2), Cuba (1), Czech (6), Denmark (9), Ecuador (1), Egypt (4), Estonia (2), Finland (8), France (29), Germany (87), Greece (22), Hungary (11), India (32), Indonesia (2), Iran (10), Ireland (6), Israel (13), Italy (124), Japan (140), Jordan (2), Kuwait (1), Lebanon (4), Lithuania (2), Malaysia (1), Mexico (11), Morocco (1), Moldova (1), Netherlands (32), New Zealand (2), Norway (13), Pakistan (2), Poland (11), Portugal (6), Romania (4), Russia (1), Saudi Arabia (3), Serbia (3), Singapore (11), Slovenia (1), South Africa (3), South Korea (46), Spain (43), Sri Lanka (1), Sweden (17), Switzerland (12), Thailand (1), Trinidad and Tobago (1), Turkey (30), United Arab Emirates (2), United Kingdom (95), United States (285), and Uruguay (1).

Phillip S Oates, *Perth*
Ross C Smith, *Sydney*
Kevin J Spring, *Brisbane*
Philip G Dinning, *Koagarah*
Christopher Christophi, *Melbourne*
Cuong D Tran, *North Adelaide*
Shan Rajendra, *Tasmania*
Rajvinder Singh, *Adelaide*
William Kemp, *Melbourne*
Phil Sutton, *Melbourne*
Richard Anderson, *Victoria*
Vance Matthews, *Melbourne*
Alexander G Heriot, *Melbourne*
Debbie Trinder, *Fremantle*
Ian C Lawrance, *Perth*
Adrian G Cummins, *Adelaide*
John K Olynyk, *Fremantle*
Alex Boussioutas, *Melbourne*
Emilia Prakoso, *Sydney*
Robert JL Fraser, *Daw Park*

Austria

Wolfgang Mikulits, *Vienna*
Alfred Gangl, *Vienna*
Dietmar Öfner, *Salzburg*
Georg Roth, *Vienna*
Herwig R Cerwenka, *Graz*
Ashraf Dahaba, *Graz*
Markus Raderer, *Vienna*
Alexander M Hirschl, *Wien*
Thomas Wild, *Kapellerfeld*
Peter Ferenci, *Vienna*
Valentin Fuhrmann, *Vienna*
Kurt Lenz, *Linz*
Markus Peck-Radosavljevic, *Vienna*
Michael Trauner, *Vienna*
Stefan Riss, *Vienna*

Belgium

Rudi Beyaert, *Gent*
Inge I Depoortere, *Leuven*
Olivier Detry, *Liège*
Benedicte Y De Winter, *Antwerp*
Etienne M Sokal, *Brussels*
Marc Peeters, *De Pintelaan*
Eddie Wisse, *Keerbergen*
Jean-Yves L Reginster, *Liège*
Mark De Ridder, *Brussel*
Freddy Penninckx, *Leuven*
Kristin Verbeke, *Leuven*
Lukas Van Oudenhove, *Leuven*
Leo van Grunsven, *Brussels*
Philip Meuleman, *Ghent*

Brazil

Heitor Rosa, *Goiania*
Roberto J Carvalho-Filho, *Sao Paulo*
Damiao Carlos Moraes Santos, *Rio de Janeiro*
Marcelo Lima Ribeiro, *Braganca Paulista*
Eduardo Garcia Vilela, *Belo Horizonte*
Jaime Natan Eisig, *São Paulo*
Andre Castro Lyra, *Salvador*
José Liberato Ferreira Caboclo, *Brazil*
Yukie Sato-Kuwabara, *São Paulo*
Raquel Rocha, *Salvador*
Paolo R Salvalaggio, *Sao Paulo*
Ana Cristina Simões e Silva, *Belo Horizonte*
Joao Batista Teixeira Rocha, *Santa Maria*

Brunei Darussalam

Vui Heng Chong, *Bandar Seri Begawan*

Bulgaria

Zahariy Krastev, *Sofia*
Mihaela Petrova, *Sofia*

Canada

Eldon Shaffer, *Calgary*
Nathalie Perreault, *Sherbrooke*
Philip H Gordon, *Montreal*
Ram Prakash Galwa, *Ottawa*
Baljinder Singh Salh, *Vancouver*
Claudia Zwingmann, *Montreal*
Alain Bitton, *Montreal*
Pingchang Yang, *Hamilton*
Michael F Byrne, *Vancouver*
Andrew L Mason, *Alberta*
John K Marshall, *Hamilton Ontario*
Kostas Pantopoulos, *Montreal*
Waliul Khan, *Ontario*
Eric M Yoshida, *Vancouver*
Geoffrey C Nguyen, *Toronto*
Devendra K Amre, *Montreal*
Tedros Bezabeh, *Winnipeg*
Wangxue Chen, *Ottawa*
Qiang Liu, *Saskatoon*

Chile

De Aretxabala Xabier, *Santiago*
Marcelo A Beltran, *La Serena*
Silvana Zanlungo, *Santiago*

China

Chi-Hin Cho, *Hong Kong*
Chun-Qing Zhang, *Jinan*
Ren Xiang Tan, *Nanjing*
Fei Li, *Beijing*
Hui-Jie Bian, *Xi'an*
Xiao-Peng Zhang, *Beijing*
Xing-Hua Lu, *Beijing*
Fu-Sheng Wang, *Beijing*
An-Gang Yang, *Xi'an*
Xiao-Ping Chen, *Wuhan*
Zong-Jie Cui, *Beijing*
Ming-Liang He, *Hong Kong*
Yuk-Tong Lee, *Hong Kong*
Qin Su, *Beijing*
Jian-Zhong Zhang, *Beijing*
Paul Kwong-Hang Tam, *Hong Kong*
Wen-Rong Xu, *Zhenjiang*
Chun-Yi Hao, *Beijing*
San-Jun Cai, *Shanghai*
Simon Law, *Hong Kong*
Yuk Him Tam, *Hong Kong*
De-Liang Fu, *Shanghai*
Eric WC Tse, *Hong Kong*
Justin CY Wu, *Hong Kong*
Nathalie Wong, *Hong Kong*
Jing Yuan Fang, *Shanghai*
Yi-Min Mao, *Shanghai*
Wei-Cheng You, *Beijing*
Xiang-Dong Wang, *Shanghai*
Xuan Zhang, *Beijing*
Zhao-Shen Li, *Shanghai*
Guang-Wen Cao, *Shanghai*
En-min Li, *Shantou*
Yu-Yuan Li, *Guangzhou*
Fook Hong Ng, *Hong Kong*
Hsiang-Fu Kung, *Hong Kong*
Wai Lun Law, *Hong Kong*
Eric CH Lai, *Hong Kong*
Jun Yu, *Hong Kong*
Ze-Guang Han, *Shanghai*
Bian zhao-xiang, *Hong Kong*
Wei-Dong Tong, *Chongqing*

Colombia

Germán Campuzano-Maya, *Medellín*

Croatia

Tamara Cacev, *Zagreb*
Marko Duvnjak, *Zagreb*

Cuba

Damian C Rodriguez, *Havana*

Czech

Milan Jirsa, *Praha*
Pavel Trunečka, *Prague*
Jan Bures, *Hradec Kralove*
Marcela Kopacova, *Hradec Kralove*
Ondrej Slaby, *Brno*
Radan Bruha, *Prague*

Denmark

Asbjørn M Drewes, *Aalborg*
Leif Percival Andersen, *Copenhagen*
Jan Mollenhauer, *Odense C*
Morten Frisch, *Copenhagen S*
Jorgen Rask-Madsen, *Skodsborg*
Morten Hylander Møller, *Holte*
Søren Rafaelsen, *Vejle*
Vibeke Andersen, *Aabenraa*
Ole Haagen Nielsen, *Herlev*

Ecuador

Fernando E Sempértegui, *Quito*

Egypt

Zeinab Nabil Ahmed Said, *Cairo*
Hussein M Atta, *El-Minia*
Asmaa Gaber Abdou, *Shebein Elkom*

Maha Maher Shehata, *Mansoura*

Estonia

Riina Salupere, *Tartu*
Tamara Vorobjova, *Tartu*

Finland

Saila Kauhanen, *Turku*
Pauli Antero Puolakkainen, *Turku*
Minna Nyström, *Helsinki*
Juhani Sand, *Tampere*
Jukka-Pekka Mecklin, *Jyvaskyla*
Lea Veijola, *Helsinki*
Kaija-Leena Kolho, *Helsinki*
Thomas Kietzmann, *Oulu*

France

Boris Guiu, *Dijon*
Baumert F Thomas, *Strasbourg*
Alain L Servin, *Châtenay-Malabry*
Patrick Marcellin, *Paris*
Jean-Jacques Tuech, *Rouen*
Francoise L Fabiani, *Angers*
Jean-Luc Faucheron, *Grenoble*
Philippe Lehours, *Bordeaux*
Stephane Supiot, *Nantes*
Lionel Bueno, *Toulouse*
Flavio Maina, *Marseille*
Paul Hofman, *Nice*
Abdel-Majid Khatib, *Paris*
Annie Schmid-Alliana, *Nice cedex 3*
Frank Zerbib, *Bordeaux Cedex*
Rene Gerolami Santandera, *Marseille*
Sabine Colnot, *Paris*
Catherine Daniel, *Lille Cedex*
Thabut Dominique, *Paris*
Laurent Huwart, *Paris*
Alain Braillon, *Amiens*
Bruno Bonaz, *Grenoble*
Evelyne Schvoerer, *Strasbourg*
M Coeffier, *Rouen*
Mathias Chamaillard, *Lille*
Hang Nguyen, *Clermont-Ferrand*
Veronique Vitton, *Marseille*
Alexis Desmoulière, *Limoges*
Juan Iovanna, *Marseille*

Germany

Hans L Tillmann, *Leipzig*
Stefan Kubicka, *Hannover*
Elke Cario, *Essen*
Hans Scherubl, *Berlin*
Harald F Teutsch, *Ulm*
Peter Konturek, *Erlangen*
Thilo Hackert, *Heidelberg*
Jurgen M Stein, *Frankfurt*
Andrej Khandoga, *Munich*
Karsten Schulmann, *Bochum*
Jutta Elisabeth Lüttges, *Riegelsberg*
Wolfgang Hagmann, *Heidelberg*
Hubert Blum, *Freiburg*
Thomas Bock, *Berlin*
Christa Buechler, *Regensburg*
Christoph F Dietrich, *Bad Mergentheim*
Ulrich R Fölsch, *Kiel*
Nikolaus Gassler, *Aachen*
Markus Gerhard, *Munich*
Dieter Glebe, *Giessen*
Klaus R Herrlinger, *Stuttgart*
Eberhard Hildt, *Berlin*
Joerg C Hoffmann, *Ludwigshafen*
Joachim Labenz, *Siegen*
Peter Malfertheiner, *Magdeburg*
Sabine Mihm, *Göttingen*
Markus Reiser, *Bochum*
Steffen Rickes, *Magdeburg*
Andreas G Schreyer, *Regensburg*
Henning Schulze-Bergkamen, *Heidelberg*
Ulrike S Stein, *Berlin*
Wolfgang R Stremmel, *Heidelberg*
Fritz von Weizsäcker, *Berlin*
Stefan Wirth, *Wuppertal*
Dean Bogoevski, *Hamburg*
Bruno Christ, *Halle/Saale*
Peter N Meier, *Hannover*
Stephan Johannes Ott, *Kiel*
Arndt Vogel, *Hannover*
Dirk Haller, *Freising*
Jens Standop, *Bonn*
Jonas Mudter, *Erlangen*
Jürgen Büning, *Lübeck*
Matthias Ocker, *Erlangen*
Joerg Trojan, *Frankfurt*
Christian Trautwein, *Aachen*
Jorg Kleeff, *Munich*
Christian Rust, *Munich*
Claus Hellerbrand, *Regensburg*
Elke Roeb, *Giessen*
Erwin Biecker, *Siegburg*
Ingmar Königsrainer, *Tübingen*
Jürgen Borlak, *Hannover*
Axel M Gressner, *Aachen*
Oliver Mann, *Hamburg*
Marty Zdichavsky, *Tübingen*
Christoph Reichel, *Bad Brückenau*
Nils Habbe, *Marburg*
Thomas Wex, *Magdeburg*
Frank Ulrich Weiss, *Greifswald*
Manfred V Singer, *Mannheim*
Martin K Schilling, *Homburg*
Philip D Hard, *Giessen*
Michael Linnebacher, *Rostock*
Ralph Graeser, *Freiburg*
Rene Schmidt, *Freiburg*
Robert Obermaier, *Freiburg*
Sebastian Mueller, *Heidelberg*
Andrea Hille, *Goettingen*
Klaus Mönkemüller, *Bottrop*
Elfriede Bollschweiler, *Köln*
Siegfried Wagner, *Deggendorf*
Dieter Schilling, *Mannheim*
Joerg F Schlaak, *Essen*
Michael Keese, *Frankfurt*
Robert Grützmann, *Dresden*
Ali Canbay, *Essen*
Dirk Domagk, *Muenster*
Jens Hoeppner, *Freiburg*
Frank Tacke, *Aachen*
Patrick Michl, *Marburg*
Alfred A Königsrainer, *Tübingen*
Kilian Weigand, *Heidelberg*
Mohamed Hassan, *Duesseldorf*
Gustav Paumgartner, *Munich*
Philipe N Khalil, *Munich*
Martin Storr, *Munich*

Greece

Andreas Larentzakis, *Athens*
Tsianos Epameinondas, *Ioannina*
Elias A Kouroumalis, *Heraklion*
Helen Christopoulou-Aletra, *Thessaloniki*
George Papatheodoridis, *Athens*
Ioannis Kanellos, *Thessaloniki*
Michael Koutsilieris, *Athens*
T Choli-Papadopoulou, *Thessaloniki*
Emanuel K Manesis, *Athens*
Evangelos Tsiambas, *Ag Paraskevi Attiki*
Konstantinos Mimidis, *Alexandroupolis*
Spilios Manolakopoulos, *Athens*
Spiros Sgouros, *Athens*
Ioannis E Koutroubakis, *Heraklion*
Stefanos Karagiannis, *Athens*
Spiros Ladas, *Athens*
Elena Vezali, *Athens*
Dina G Tiniakos, *Athens*
Ekaterini Chatzaki, *Alexandroupolis*
Dimitrios Roukos, *Ioannina*
George Sgourakis, *Athens*
Maroulio Talieri, *Athens*

Hungary

Peter L Lakatos, *Budapest*
Yvette Mándi, *Szeged*
Ferenc Sipos, *Budapest*
György M Buzás, *Budapest*
László Czakó, *Szeged*
Peter Hegyi, *Szeged*
Zoltan Rakonczay, *Szeged*
Gyula Farkas, *Szeged*
Zsuzsa Szondy, *Debrecen*
Gabor Veres, *Budapest*
Zsuzsa Schaff, *Budapest*

India

Philip Abraham, *Mumbai*
Sri P Misra, *Allahabad*
Ramesh Roop Rai, *Jaipur*
Nageshwar D Reddy, *Hyderabad*
Rakesh Kumar Tandon, *New Delhi*
Jai Dev Wig, *Chandigarh*
Uday C Ghoshal, *Lucknow*
Pramod Kumar Garg, *New Delhi*
Barjesh Chander Sharma, *New Delhi*
Gopal Nath, *Varanasi*
Bhupendra Kumar Jain, *Delhi*
Devinder Kumar Dhawan, *Chandigarh*
Ashok Kumar, *Lucknow*
Benjamin Perakath, *Tamil Nadu*
Debidas Ghosh, *Midnpore*
Pankaj Garg, *Panchkula*
Samiran Nundy, *New Delhi*
Virendra Singh, *Chandigarh*
Bikash Medhi, *Chandigarh*
Radha K Dhiman, *Chandigarh*
Vandana Panda, *Mumbai*
Vineet Ahuja, *New Delhi*
SV Rana, *Chandigarh*

Deepak N Amarapurkar, *Mumbai*
Abhijit Chowdhury, *Kolkata*
Jasbir Singh, *Kurukshetra*
B Mittal, *Lucknow*
Sundeep Singh Saluja, *New Delhi*
Pradyumna Kumar Mishra, *Mumbai*
Runu Chakravarty, *Kolkata*
Nagarajan Perumal, *New Delhi*

Indonesia

David handojo Muljono, *Jakarta*
Andi Utama, *Tangerang*

Iran

Seyed-Moayed Alavian, *Tehran*
Reza Malekzadeh, *Tehran*
Peyman Adibi, *Isfahan*
Alireza Mani, *Tehran*
Seyed Mohsen Dehghani, *Shiraz*
Mohammad Abdollahi, *Tehran*
Majid Assadi, *Bushehr*
Arezoo Aghakhani, *Tehran*
Marjan Mohammadi, *Tehran*
Fariborz Mansour-Ghanaei, *Rasht*

Ireland

Ross McManus, *Dublin*
Billy Bourke, *Dublin*
Catherine Greene, *Dublin*
Ted Dinan, *Cork*
Marion Rowland, *Dublin*

Israel

Abraham R Eliakim, *Haifa*
Simon Bar-Meir, *Tel Hashomer*
Ami D Sperber, *Beer-Sheva*
Boris Kirshtein, *Beer Sheva*
Mark Pines, *Bet Dagan*
Menachem Moshkowitz, *Tel-Aviv*
Ron Shaoul, *Haifa*
Shmuel Odes, *Beer Sheva*
Sigal Fishman, *Tel Aviv*
Alexander Becker, *Afula*
Assy Nimer, *Safed*
Eli Magen, *Ashdod*
Amir Shlomai, *Tel-Aviv*

Italy

Mauro Bortolotti, *Bologna*
Gianlorenzo Dionigi, *Varese*
Fiorucci Stefano, *Perugia*
Roberto Berni Canani, *Naples*
Ballarin Roberto, *Modena*
Bruno Annibale, *Roma*
Vincenzo Stanghellini, *Bologna*
Giovanni B Gaeta, *Napoli*
Claudio Bassi, *Verona*
Mauro Bernardi, *Bologna*
Giuseppe Chiarioni, *Valeggio*
Michele Cicala, *Rome*
Dario Conte, *Milano*
Francesco Costa, *Pisa*
Giovanni D De Palma, *Naples*
Giammarco Fava, *Ancona*
Francesco Feo, *Sassari*
Edoardo G Giannini, *Genoa*
Fabio Grizzi, *Milan*
Salvatore Gruttadauria, *Palermo*
Pietro Invernizzi, *Milan*
Ezio Laconi, *Cagliari*
Giuseppe Montalto, *Palermo*
Giovanni Musso, *Torino*
Gerardo Nardone, *Napoli*
Valerio Nobili, *Rome*
Raffaele Pezzilli, *Bologna*
Alberto Piperno, *Monza*
Anna C Piscaglia, *Roma*
Piero Portincasa, *Bari*
Giovanni Tarantino, *Naples*
Cesare Tosetti, *Porretta Terme*
Alessandra Ferlini, *Ferrara*
Alessandro Ferrero, *Torino*
Donato F Altomare, *Bari*
Giovanni Milito, *Rome*
Giuseppe Sica, *Rome*
Guglielmo Borgia, *Naples*
Giovanni Latella, *L'Aquila*
Salvatore Auricchio, *Naples*
Alberto Biondi, *Rome*
Alberto Tommasini, *Trieste*
Antonio Basoli, *Roma*
Giuliana Decorti, *Trieste*
Marco Silano, *Roma*
Michele Reni, *Milan*
Pierpaolo Sileri, *Rome*
Achille Iolascon, *Naples*
Alessandro Granito, *Bologna*
Angelo A Izzo, *Naples*
Giuseppe Currò, *Messina*
Pier Mannuccio Mannucci, *Milano*
Marco Vivarelli, *Bologna*
Massimo Levrero, *Rome*
Massimo Rugge, *Padova*
Paolo Angeli, *Padova*
Silvio Danese, *Milano*
Antonello Trecca, *Rome*
Antonio Gasbarrini, *Rome*
Cesare Ruffolo, *Treviso*
Massimo Falconi, *Verona*
Fausto Catena, *Bologna*
Francesco Manguso, *Napoli*
Giancarlo Mansueto, *Verona*
Luca Morelli, *Trento*
Marco Scarpa, *Padova*
Mario M D'Elios, *Florence*
Francesco Luzza, *Catanzaro*
Franco Roviello, *Siena*
Guido Torzilli, *Rozzano Milano*
Luca Frulloni, *Verona*
Lucia Malaguarnera, *Catania*
Lucia Ricci Vitiani, *Rome*
Mara Massimi, *L'Aquila*
Mario Pescatori, *Rome*
Mario Rizzetto, *Torino*
Mirko D'Onofrio, *Verona*
Nadia Peparini, *Rome*
Paola De Nardi, *Milan*
Paolo Aurello, *Rome*
Piero Amodio, *Padova*
Riccardo Nascimbeni, *Brescia*
Vincenzo Villanacci, *Brescia*
Vittorio Ricci, *Pavia*
Silvia Fargion, *Milan*
Luigi Bonavina, *Milano*
Oliviero Riggio, *Rome*
Fabio Pace, *Milano*
Gabrio Bassotti, *Perugia*
Giulio Marchesini, *Bologna*
Roberto de Franchis, *Milano*
Giovanni Monteleone, *Rome*
C armelo Scarpignato, *Parma*
Luca VC Valenti, *Milan*
Urgesi Riccardo, *Rome*
Marcello Persico, *Naples*
Antonio Moschetta, *Bari*
Luigi Muratori, *Bologna*
Angelo Zullo, *Roma*
Vito Annese, *Florence*
Simone Lanini, *Rome*
Alessandro Grasso, *Savona*
Giovanni Targher, *Verona*
Domenico Girelli, *Verona*
Alessandro Cucchetti, *Bologna*
Fabio Marra, *Florence*
Michele Milella, *Rome*
Francesco Franceschi, *Rome*
Giuseppina De Petro, *Brescia*
Salvatore Leonardi, *Catania*
Cristiano Simone, *Santa Maria Imbaro*
Bernardino Rampone, *Salerno*
Francesco Crea, *Pisa*
Walter Fries, *Messina*
Antonio Craxì, *Palermo*
Gerardo Rosati, *Potenza*
Mario Guslandi, *Milano*
Gianluigi Giannelli, *Bari*
Paola Loria, *Modena*
Paolo Sorrentino, *Avellino*
Armando Santoro, *Rozzano*
Gabriele Grassi, *Trieste*
Antonio Orlacchio, *Rome*

Japan

Tsuneo Kitamura, *Chiba*
Katsutoshi Yoshizato, *Higashihiroshima*
Masahiro Arai, *Tokyo*
Shinji Tanaka, *Hiroshima*
Keiji Hirata, *Kitakyushu*
Yoshio Shirai, *Niigata*
Susumu Ohmada, *Maebashi*
Kenichi Ikejima, *Tokyo*
Masatoshi Kudo, *Osaka*
Yoshiaki Murakami, *Hiroshima*
Masahiro Tajika, *Nagoya*
Kentaro Yoshika, *Toyoake*
Kyoichi Adachi, *Izumo*
Yasushi Adachi, *Sapporo*
Takafumi Ando, *Nagoya*
Akira Andoh, *Otsu*
Hitoshi Asakura, *Tokyo*
Mitsuhiro Fujishiro, *Tokyo*
Toru Hiyama, *Higashihiroshima*
Yutaka Inagaki, *Kanagawa*
Hiromi Ishibashi, *Nagasaki*
Shunji Ishihara, *Izumo*
Toru Ishikawa, *Niigata*
Yoshiaki Iwasaki, *Okayama*
Terumi Kamisawa, *Tokyo*

Norihiro Kokudo, *Tokyo*
Shin Maeda, *Tokyo*
Yasushi Matsuzaki, *Ibaraki*
Kenji Miki, *Tokyo*
Hiroto Miwa, *Hyogo*
Yoshiharu Motoo, *Kanazawa*
Kunihiko Murase, *Tusima*
Atsushi Nakajima, *Yokohama*
Yuji Naito, *Kyoto*
Hisato Nakajima, *Tokyo*
Hiroki Nakamura, *Yamaguchi*
Shotaro Nakamura, *Fukuoka*
Mikio Nishioka, *Niihama*
Hirohide Ohnishi, *Akita*
Kazuichi Okazaki, *Osaka*
Morikazu Onji, *Ehime*
Satoshi Osawa, *Hamamatsu*
Hidetsugu Saito, *Tokyo*
Yutaka Saito, *Tokyo*
Yasushi Sano, *Kobe*
Tomohiko Shimatani, *Kure*
Yukihiro Shimizu, *Toyama*
Shinji Shimoda, *Fukuoka*
Masayuki Sho, *Nara*
Hidekazu Suzuki, *Tokyo*
Shinji Togo, *Yokohama*
Satoshi Yamagiwa, *Niigata*
Takayuki Yamamoto, *Yokkaichi*
Hiroshi Yoshida, *Tokyo*
Norimasa Yoshida, *Kyoto*
Akihito Nagahara, *Tokyo*
Hiroaki Takeuchi, *Kochi*
Keiji Ogura, *Tokyo*
Kotaro Miyake, *Tokushima*
Mitsunori Yamakawa, *Yamagata*
Naoaki Sakata, *Sendai*
Naoya Kato, *Tokyo*
Satoshi Mamori, *Hyogo*
Shogo Kikuchi, *Aichi*
Shoichiro Sumi, *Kyoto*
Susumu Ikehara, *Osaka*
Taketo Yamaguchi, *Chiba*
Tokihiko Sawada, *Tochigi*
Tomoharu Yoshizumi, *Fukuoka*
Toshiyuki Ishiwata, *Tokyo*
Yasuhiro Fujino, *Akashi*
Yasuhiro Koga, *Isehara city*
Yoshihisa Takahashi, *Tokyo*
Yoshitaka Takuma, *Okayama*
Yutaka Yata, *Maebashi-city*
Itaru Endo, *Yokohama*
Kazuo Chijiiwa, *Miyazaki*
Kouhei Fukushima, *Sendai*
Masahiro Iizuka, *Akita*
Mitsuyoshi Urashima, *Tokyo*
Munechika Enjoji, *Fukuoka*
Takashi Kojima, *Sapporo*
Takumi Kawaguchi, *Kurume*
Yoshiyuki Ueno, *Sendai*
Yuichiro Eguchi, *Saga*
Akihiro Tamori, *Osaka*
Atsushi Masamune, *Sendai*
Atsushi Tanaka, *Tokyo*
Hitoshi Tsuda, *Tokyo*
Takashi Kobayashi, *Tokyo*
Akimasa Nakao, *Nagogya*
Hiroyuki Uehara, *Osaka*
Masahito Uemura, *Kashihara*
Satoshi Tanno, *Sapporo*
Toshinari Takamura, *Kanazawa*
Yohei Kida, *Kainan*

Masanori Hatakeyama, *Tokyo*
Satoru Kakizaki, *Gunma*
Shuhei Nishiguchi, *Hyogo*
Yuichi Yoshida, *Osaka*
Manabu Morimoto, *Japan*
Mototsugu Kato, *Sapporo*
Naoki Ishii, *Tokyo*
Noriko Nakajima, *Tokyo*
Nobuhiro Ohkohchi, *Tsukuba*
Takanori Kanai, *Tokyo*
Kenichi Goda, *Tokyo*
Mitsugi Shimoda, *Mibu*
Zenichi Morise, *Nagoya*
Hitoshi Yoshiji, *Kashihara*
Takahiro Nakazawa, *Nagoya*
Utaroh Motosugi, *Yamanashi*
Nobuyuki Matsuhashi, *Tokyo*
Yasuhiro Kodera, *Nagoya*
Takayoshi Ito, *Tokyo*
Yasuhito Tanaka, *Nagoya*
Haruhiko Sugimura, *Hamamatsu*
Hiroki Yamaue, *Wakayama*
Masao Ichinose, *Wakayama*
Takaaki Arigami, *Kagoshima*
Nobuhiro Zaima, *Nara*
Naoki Tanaka, *Matsumoto*
Satoru Motoyama, *Akita*
Tomoyuki Shibata, *Toyoake*
Tatsuya Ide, *Kurume*
Tsutomu Fujii, *Nagoya*
Osamu Kanauchi, *Toky*
Atsushi Irisawa, *Aizuwakamatsu*
Hikaru Nagahara, *Tokyo*
Keiji Hanada, *Onomichi*
Keiichi Mitsuyama, *Fukuoka*
Shin Maeda, *Yokohama*
Takuya Watanabe, *Niigata*
Toshihiro Mitaka, *Sapporo*
Yoshiki Murakami, *Kyoto*
Tadashi Shimoyama, *Hirosaki*

Jordan

Ismail Matalka, *Irbid*
Khaled Jadallah, *Irbid*

Kuwait

Islam Khan, *Safat*

Lebanon

Bassam N Abboud, *Beirut*
Rami Moucari, *Beirut*
Ala I Sharara, *Beirut*
Rita Slim, *Beirut*

Lithuania

Giedrius Barauskas, *Kaunas*
Limas Kupcinskas, *Kaunas*

Malaysia

Andrew Seng Boon Chua, *Ipoh*

Mexico

Saúl Villa-Trevio, *Mexico*
Omar Vergara-Fernandez, *Mexico*
Diego Garcia-Compean, *Monterrey*
Arturo Panduro, *Jalisco*
Miguel Angel Mercado, *Distrito Federal*
Richard A Awad, *Mexico*
Aldo Torre Delgadillo, *Mexico*
Paulino Martínez Hernández Magro, *Celaya*
Carlos A Aguilar-Salinas, *Mexico*
Jesus K Yamamoto-Furusho, *Mexico*

Morocco

Samir Ahboucha, *Khouribga*

Moldova

Igor Mishin, *Kishinev*

Netherlands

Ulrich Beuers, *Amsterdam*
Albert Frederik Pull ter Gunne, *Tilburg*
Jantine van Baal, *Heidelberglaan*
Wendy Wilhelmina Johanna de Leng, *Utrecht*
Gerrit A Meijer, *Amsterdam*
Lee Bouwman, *Leiden*
J Bart A Crusius, *Amsterdam*
Frank Hoentjen, *Haarlem*
Servaas Morré, *Amsterdam*
Chris JJ Mulder, *Amsterdam*
Paul E Sijens, *Groningen*
Karel van Erpecum, *Utrecht*
BW Marcel Spanier, *Arnhem*
Misha Luyer, *Sittard*
Pieter JF de Jonge, *Rotterdam*
Robert Christiaan Verdonk, *Groningen*
John Plukker, *Groningen*
Maarten Tushuizen, *Amsterdam*
Wouter de Herder, *Rotterdam*
Erwin G Zoetendal, *Wageningen*
Robert J de Knegt, *Rotterdam*
Albert J Bredenoord, *Nieuwegein*
Annemarie de Vries, *Rotterdam*
Astrid van der Velde, *Ede*
Lodewijk AA Brosens, *Utrecht*
James CH Hardwick, *Leiden*
Loes van Keimpema, *Nijmegen*
WJ de Jonge, *Amsterdam*
Zuzana Zelinkova, *Rotterdam*
LN van Steenbergen, *Eindhoven*
Frank G Schaap, *Amsterdam*
Jeroen Maljaars, *Leiden*

New Zealand

Andrew S Day, *Christchurch*
Max S Petrov, *Auckland*

Norway

Espen Melum, *Oslo*

Trine Olsen, *Tromsø*
Eyvind J Paulssen, *Tromsø*
Rasmus Goll, *Tromsø*
Asle W Medhus, *Oslo*
Jon Arne Søreide, *Stavanger*
Kjetil Soreide, *Stavanger*
Reidar Fossmark, *Trondheim*
Trond Peder Flaten, *Trondheim*
Olav Dalgard, *Oslo*
Ole Høie, *Arendal*
Magdy El-Salhy, *Bergen*
Jørgen Valeur, *Oslo*

Pakistan

Shahab Abid, *Karachi*
Syed MW Jafri, *Karachi*

Poland

Beata Jolanta Jablońska, *Katowice*
Halina Cichoż-Lach, *Lublin*
Tomasz Brzozowski, *Cracow*
Hanna Gregorek, *Warsaw*
Marek Hartleb, *Katowice*
Stanislaw J Konturek, *Krakow*
Andrzej Dabrowski, *Bialystok*
Jan Kulig, *Kraków*
Julian Swierczynski, *Gdansk*
Marek Bebenek, *Wroclaw*
Dariusz M Lebensztejn, *Bialystok*

Portugal

Ricardo Marcos, *Porto*
Guida Portela-Gomes, *Estoril*
Ana Isabel Lopes, *Lisboa Codex*
Raquel Almeida, *Porto*
Rui Tato Marinho, *Lisbon*
Ceu Figueiredo, *Porto*

Romania

Dan L Dumitrascu, *Cluj*
Adrian Saftoiu, *Craiova*
Andrada Seicean, *Cluj-Napoca*
Anca Trifan, *Iasi*

Russia

Vasiliy I Reshetnyak, *Moscow*

Saudi Arabia

Ibrahim A Al Mofleh, *Riyadh*
Abdul-Wahed Meshikhes, *Qatif*
Faisal Sanai, *Riyadh*

Serbia

Tamara M Alempijevic, *Belgrade*
Dusan M Jovanovic, *Sremska Kamenica*
Zoran Krivokapic, *Belgrade*

Singapore

Brian Kim Poh Goh, *Singapore*
Khek-Yu Ho, *Singapore*
Fock Kwong Ming, *Singapore*
Francis Seow-Choen, *Singapore*
Kok Sun Ho, *Singapore*
Kong Weng Eu, *Singapore*
Madhav Bhatia, *Singapore*
London Lucien Ooi, *Singapore*
Wei Ning Chen, *Singapore*
Richie Soong, *Singapore*
Kok Ann Gwee, *Singapore*

Slovenia

Matjaz Homan, *Ljubljana*

South Africa

Rosemary Joyce Burnett, *Pretoria*
Michael Kew, *Cape Town*
Roland Ndip, *Alice*

South Korea

Byung Chul Yoo, *Seoul*
Jae J Kim, *Seoul*
Jin-Hong Kim, *Suwon*
Marie Yeo, *Suwon*
Jeong Min Lee, *Seoul*
Eun-Yi Moon, *Seoul*
Joong-Won Park, *Goyang*
Hoon Jai Chun, *Seoul*
Myung-Gyu Choi, *Seoul*
Sang Kil Lee, *Seoul*
Sang Yeoup Lee, *Gyeongsangnam-do*
Won Ho Kim, *Seoul*
Dae-Yeul Yu, *Daejeon*
Donghee Kim, *Seoul*
Sang Geon Kim, *Seoul*
Sun Pyo Hong, *Geonggi-do*
Sung-Gil Chi, *Seoul*
Yeun-Jun Chung, *Seoul*
Ki-Baik Hahm, *Incheon*
Ji Kon Ryu, *Seoul*
Kyu Taek Lee, *Seoul*
Yong Chan Lee, *Seoul*
Seong Gyu Hwang, *Seongnam*
Seung Woon Paik, *Seoul*
Sung Kim, *Seoul*
Hong Joo Kim, *Seoul*
Hyoung-Chul Oh, *Seoul*
Nayoung Kim, *Seongnam-si*
Sang Hoon Ahn, *Seoul*
Seon Hahn Kim, *Seoul*
Si Young Song, *Seoul*
Young-Hwa Chung, *Seoul*
Hyo-Cheol Kim, *Seoul*
Kwang Jae Lee, *Swon*
Sang Min Park, *Seoul*
Young Chul Kim, *Seoul*
Do Hyun Park, *Seoul*
Dae Won Jun, *Seoul*
Dong Wan Seo, *Seoul*
Soon-Sun Hong, *Incheon*
Hoguen Kim, *Seoul*
Ho-Young Song, *Seoul*
Joo-Ho Lee, *Seoul*
Jung Eun Lee, *Seoul*
Jong H Moon, *Bucheon*

Spain

Eva Vaquero, *Barcelona*
Andres Cardenas, *Barcelona*
Laureano Fernández-Cruz, *Barcelona*
Antoni Farré, *Spain*
Maria-Angeles Aller, *Madrid*
Raul J Andrade, *Málaga*
Fernando Azpiroz, *Barcelona*
Josep M Bordas, *Barcelona*
Antoni Castells, *Barcelona*
Vicente Felipo, *Valencia*
Isabel Fabregat, *Barcelona*
Angel Lanas, *Zaragoza*
Juan-Ramón Larrubia, *Guadalajara*
María IT López, *Jaén*
Jesús M Prieto, *Pamplona*
Mireia Miquel, *Sabadell*
Ramon Bataller, *Barcelona*
Fernando J Corrales, *Pamplona*
Julio Mayol, *Madrid*
Matias A Avila, *Pamplona*
Juan Macías, *Seville*
Juan Carlos Laguna Egea, *Barcelona*
Juli Busquets, *Barcelona*
Belén Beltrán, *Valencia*
José Manuel Martin-Villa, *Madrid*
Lisardo Boscá, *Madrid*
Luis Grande, *Barcelona*
Pedro Lorenzo Majano Rodriguez, *Madrid*
Adolfo Benages, *Valencia*
Domínguez-Muñoz JE, *Santiago de Compostela*
Gloria González Aseguinolaza, *Navarra*
Javier Martin, *Granada*
Luis Bujanda, *San Sebastián*
Matilde Bustos, *Pamplona*
Luis Aparisi, *Valencia*
José Julián calvo Andrés, *Salamanca*
Benito Velayos, *Valladolid*
Javier Gonzalez-Gallego, *León*
Ruben Ciria, *Córdoba*
Francisco Rodriguez-Frias, *Barcelona*
Manuel Romero-Gómez, *Sevilla*
Albert Parés, *Barcelona*
Joan Roselló-Catafau, *Barcelona*

Sri Lanka

Arjuna De Silva, *Kelaniya*

Sweden

Stefan G Pierzynowski, *Lund*
Hanns-Ulrich Marschall, *Stockholm*
Lars A Pahlman, *Uppsala*
Helena Nordenstedt, *Stockholm*
Bobby Tingstedt, *Lund*
Evangelos Kalaitzakis, *Gothenburg*
Lars Erik Agréus, *Huddinge*
Annika Lindblom, *Stockholm*

Roland Andersson, *Lund*
Zongli Zheng, *Stockholm*
Mauro D'Amato, *Huddinge*
Greger Lindberg, *Stockholm*
Pär Erik Myrelid, *Linköping*
Sara Lindén, *Göteborg*
Sara Regnér, *Malmö*
Åke Nilsson, *Lund*

Switzerland

Jean L Frossard, *Geneva*
Andreas Geier, *Zürich*
Bruno Stieger, *Zürich*
Pascal Gervaz, *Geneva*
Paul M Schneider, *Zurich*
Felix Stickel, *Berne*
Fabrizio Montecucco, *Geneva*
Inti Zlobec, *Basel*
Michelangelo Foti, *Geneva*
Pascal Bucher, *Geneva*
Andrea De Gottardi, *Berne*
Christian Toso, *Geneva*

Thailand

Weekitt Kittisupamongkol, *Bangkok*

Trinidad and Tobago

Shivananda Nayak, *Mount Hope*

Turkey

Tarkan Karakan, *Ankara*
Yusuf Bayraktar, *Ankara*
Ahmet Tekin, *Mersin*
Aydin Karabacakoglu, *Konya*
Osman C Ozdogan, *Istanbul*
Özlem Yilmaz, *Izmir*
Bülent Salman, *Ankara*
Can GONEN, *Kutahya*
Cuneyt Kayaalp, *Malatya*
Ekmel Tezel, *Ankara*
Eren Ersoy, *Ankara*
Hayrullah Derici, *Balıkesir*
Mehmet Refik Mas, *Etlik-Ankara*
Sinan Akay, *Tekirdag*
A Mithat Bozdayi, *Ankara*
Metin Basaranoglu, *Istanbul*
Mesut Tez, *Ankara*
Orhan Sezgin, *Mersin*
Mukaddes Esrefoglu, *Malatya*
Ilker Tasci, *Ankara*
Kemal Kismet, *Ankara*
Selin Kapan, *Istanbul*
Seyfettin Köklü, *Ankara*
Murat Sayan, *Kocaeli*
Sabahattin Kaymakoglu, *Istanbul*
Yucel Ustundag, *Zonguldak*
Can Gonen, *Istanbul*
Yusuf Yilmaz, *Istanbul*
Müge Tecder-Ünal, *Ankara*
İlhami Yüksel, *Ankara*

United Arab Emirates

Fikri M Abu-Zidan, *Al-Ain*
Sherif M Karam, *Al-Ain*

United Kingdom

Anastasios Koulaouzidis, *Edinburgh*
Sylvia LF Pender, *Southampton*
Hong-Xiang Liu, *Cambridge*
William Dickey, *Londonderry*
Simon D Taylor-Robinson, *London*
James Neuberger, *Birmingham*
Frank I Tovey, *London*
Kevin Robertson, *Glasgow*
Chew Thean Soon, *Manchester*
Geoffrey Burnstock, *London*
Vamsi R Velchuru, *United Kingdom*
Simon Afford, *Birmingham*
Navneet K Ahluwalia, *Stockport*
Lesley A Anderson, *Belfast*
Anthony TR Axon, *Leeds*
Jim D Bell, *London*
Alastair D Burt, *Newcastle*
Tatjana Crnogorac-Jurcevic, *London*
Daniel R Gaya, *Edinburgh*
William Greenhalf, *Liverpool*
Indra N Guha, *Southampton*
Stefan G Hübscher, *Birmingham*
Robin Hughes, *London*
Pali Hungin, *Stockton*
Janusz AZ Jankowski, *Oxford*
Peter Karayiannis, *London*
Patricia F Lalor, *Birmingham*
Giorgina Mieli-Vergani, *London*
D Mark Pritchard, *Liverpool*
Marco Senzolo, *Padova*
Roger Williams, *London*
M H Ahmed, *Southampton*
Christos Paraskeva, *Bristol*
Emad M El-Omar, *Aberdeen*
A M El-Tawil, *Birmingham*
Anne McCune, *Bristol*
Charles B Ferguson, *Belfast*
Chin Wee Ang, *Liverpool*
Clement W Imrie, *Glasgow*
Dileep N Lobo, *Nottingham*
Graham MacKay, *Glasgow*
Guy Fairbairn Nash, *Poole*
Ian Lindsey, *Oxford*
Jason CB Goh, *Birmingham*
Jeremy FL Cobbold, *London*
Julian RF Walters, *London*
Jamie Murphy, *London*
John Beynon, *Swansea*
John B Schofield, *Kent*
Anil George, *London*
Aravind Suppiah, *East Yorkshire*
Basil Ammori, *Salford*
Catherine Walter, *Cheltenham*
Chris Briggs, *Sheffield*
Jeff Butterworth, *Shrewsbury*
Nawfal Hussein, *Nottingham*
Patrick O'Dwyer, *Glasgow*
Rob Glynne-Jones, *Northwood*
Sharad Karandikar, *Birmingham*
Venkatesh Shanmugam, *Derby*
Yeng S Ang, *Wigan*
Alberto Quaglia, *London*
Andrew Fowell, *Southampton*
Gianpiero Gravante, *Leicester*
Piers Gatenby, *London*
Kondragunta Rajendra Prasad, *Leeds*
Sunil Dolwani, *Cardiff*
Andrew McCulloch Veitch, *Wolverhampton*
Brian Green, *Belfast*
Noriko Suzuki, *Middlesex*
Richard Parker, *North Staffordshire*
Shahid A Khan, *London*
Akhilesh B Reddy, *Cambridge*
Jean E Crabtree, *Leeds*
John S Leeds, *Sheffield*
Paul Sharp, *London*
Sumita Verma, *Brighton*
Thamara Perera, *Birmingham*
Donald Campbell McMillan, *Glasgow*
Kathleen B Bamford, *London*
Helen Coleman, *Belfast*
Eyad Elkord, *Manchester*
Mohammad Ilyas, *Nottingham*
Simon R Carding, *Norwich*
Ian Chau, *Sutton*
Claudio Nicoletti, *Norwich*
Hendrik-Tobias Arkenau, *London*
Muhammad Imran Aslam, *Leicester*
Giuseppe Orlando, *Oxford*
John S Leeds, *Aberdeen*
S Madhusudan, *Nottingham*
Amin Ibrahim Amin, *Dunfermline*
David C Hay,*Edinburgh*
Alan Burns, *London*

United States

Tauseef Ali, *Oklahoma City*
George Y Wu, *Farmington*
Josef E Fischer, *Boston*
Thomas Clancy, *Boston*
John Morton, *Stanford*
Luca Stocchi, *Cleveland*
Kevin Michael Reavis, *Orange*
Shiu-Ming Kuo, *Buffalo*
Gary R Lichtenstein, *Philadelphia*
Natalie J Torok, *Sacramento*
Scott A Waldman, *Philadelphia*
Georgios Papachristou, *Pittsburgh*
Carla W Brady, *Durham*
Robert CG Martin, *Louisville*
Eugene P Ceppa, *Durham*
Shashi Bala, *Worcester*
Imran Hassan, *Springfield*
Klaus Thaler, *Columbia*
Andreas M Kaiser, *Los Angeles*
Shawn D Safford, *Norfolk*
Massimo Raimondo, *Jacksonville*
Kazuaki Takabe, *Richmond VA*
Stephen M Kavic, *Baltimore*
T Clark Gamblin, *Pittsburgh*
BS Anand, *Houston*
Ananthanarayanan M, *New York*
Anthony J Bauer, *Pittsburgh*
Edmund J Bini, *New York*
Xian-Ming Chen, *Omaha*
Ramsey Chi-man Cheung, *Palo Alto*
Parimal Chowdhury, *Arkansas*
Mark J Czaja, *New York*

World Journal of Gastroenterology

Contents

Weekly Volume 19 Number 43 November 21, 2013

EDITORIAL

7489 Challenges in diagnosing adhesive small bowel obstruction
van Oudheusden TR, Aerts BAC, de Hingh IHJT, Luyer MDP

FIELD OF VISION

7494 High intensity focused ultrasound, liver disease and bridging therapy
Luigi M

TOPIC HIGHLIGHT

7500 Role of innate immunity in the development of hepatocellular carcinoma
Aravalli RN

7515 Bridging and downstaging treatments for hepatocellular carcinoma in patients on the waiting list for liver transplantation
Pompili M, Francica G, Ponziani FR, Iezzi R, Avolio AW

7531 Enteric bacterial proteases in inflammatory bowel disease- pathophysiology and clinical implications
Carroll IM, Maharshak N

7544 Beyond white light endoscopy: The role of optical biopsy in inflammatory bowel disease
Liu J, Dlugosz A, Neumann H

7552 Mucosal healing and deep remission: What does it mean?
Rogler G, Vavricka S, Schoepfer A, Lakatos PL

7561 Has the risk of colorectal cancer in inflammatory bowel disease decreased?
Andersen NN, Jess T

7569 Autoantibodies and an immune-based rat model of inflammatory bowel disease
Esmaily H, Sanei Y, Abdollahi M

7577 *Clostridium difficile* and inflammatory bowel disease: Role in pathogenesis and implications in treatment
Nitzan O, Elias M, Chazan B, Raz R, Saliba W

Contents

World Journal of Gastroenterology
Volume 19 Number 43 November 21, 2013

REVIEW

7586 Extravascular use of drug-eluting beads: A promising approach in compartment-based tumor therapy
Binder S, Lewis AL, Löhr JM, Keese M

7594 Role of sirtuins in ischemia-reperfusion injury
Pantazi E, Zaouali MA, Bejaoui M, Folch-Puy E, Ben Abdennebi H, Roselló-Catafau J

7603 Rare cystic liver lesions: A diagnostic and managing challenge
Bakoyiannis A, Delis S, Triantopoulou C, Dervenis C

7620 Branched-chain amino acids in liver diseases
Tajiri K, Shimizu Y

MINIREVIEWS

7630 Liver diseases in pregnancy: Diseases not unique to pregnancy
Almashhrawi AA, Ahmed KT, Rahman RN, Hammoud GM, Ibdah JA

7639 Liver diseases in pregnancy: Diseases unique to pregnancy
Ahmed KT, Almashhrawi AA, Rahman RN, Hammoud GM, Ibdah JA

7647 Liver diseases in pregnancy: Liver transplantation in pregnancy
Hammoud GM, Almashhrawi AA, Ahmed KT, Rahman R, Ibdah JA

7652 Use of exclusive enteral nutrition in adults with Crohn's disease: A review
Wall CL, Day AS, Gearry RB

7661 Diagnosis of IgG4-related sclerosing cholangitis
Nakazawa T, Naitoh I, Hayashi K, Miyabe K, Simizu S, Joh T

ORIGINAL ARTICLE

7671 Efficacy of switching to telbivudine plus adefovir in suboptimal responders to lamivudine plus adefovir
Park H, Park JY, Kim SU, Kim DY, Han KH, Chon CY, Ahn SH

7680 8-bromo-7-methoxychrysin inhibits properties of liver cancer stem cells *via* downregulation of β-catenin
Quan MF, Xiao LH, Liu ZH, Guo H, Ren KQ, Liu F, Cao JG, Deng XY

BRIEF ARTICLE

7696 Laparoscopy-assisted percutaneous endoscopic gastrostomy enables enteral nutrition even in patients with distorted anatomy
Hermanowicz A, Matuszczak E, Komarowska M, Jarocka-Cyrta E, Wojnar J, Debek W, Matysiak K, Klek S

7701 Is early limited surgery associated with a more benign disease course in Crohn's disease?
Golovics PA, Lakatos L, Nagy A, Pandur T, Szita I, Balogh M, Molnar C, Komaromi E, Lovasz BD, Mandel M, Veres G, Kiss LS, Vegh Z, Lakatos PL

7711 Dendritic cell co-stimulatory and co-inhibitory markers in chronic HCV: An Egyptian study
Fouad H, El Raziky MS, Abdel Aziz RA, Sabry D, Abdel Aziz GM, Ewais M, Sayed AR

7719 Characteristics of nonvariceal upper gastrointestinal hemorrhage in patients with chronic kidney disease
Bang CS, Lee YS, Lee YH, Sung H, Park HJ, Kim HS, Kim JB, Baik GH, Kim YS, Yoon JH, Kim DJ, Suk KT

7726 Events associated with apoptotic effect of *p*-Coumaric acid in HCT-15 colon cancer cells
Jaganathan SK, Supriyanto E, Mandal M

7735 Screening of *SLC25A13* mutation in the Thai population
Wongkittichote P, Sukasem C, Kikuchi A, Aekplakorn W, Jensen LT, Kure S, Wattanasirichaigoon D

7743 Single-incision *vs* three-incision laparoscopic cholecystectomy for complicated and uncomplicated acute cholecystitis
Chuang SH, Chen PH, Chang CM, Lin CS

7751 Transanal natural orifice specimen extraction for laparoscopic anterior resection in rectal cancer
Han FH, Hua LX, Zhao Z, Wu JH, Zhan WH

7758 MicroRNA-143 suppresses gastric cancer cell growth and induces apoptosis by targeting COX-2
Wu XL, Cheng B, Li PY, Huang HJ, Zhao Q, Dan ZL, Tian D, Zhang P

7766 PU.1-silenced dendritic cells prolong allograft survival in rats receiving intestinal transplantation
Xu XW, Ding BW, Zhu CR, J i W, Li JS

7772 Biliary casts after liver transplantation: Morphology and biochemical analysis
Yang YL, Zhang C, Lin MJ, Shi LJ, Zhang HW, Li JY, Yu Q

7778 Amelioration of carbon tetrachloride-induced cirrhosis and portal hypertension in rat using adenoviral gene transfer of Akt
Deng G, Huang XJ, Luo HW, Huang FZ, Liu XY, Wang YH

7788 Insulin-like growth factor-1 induces lymphangiogenesis and facilitates lymphatic metastasis in colorectal cancer
Li ZJ, Ying XJ, Chen HL, Ye PJ, Chen ZL, Li G, Jiang HF, Liu J, Zhou SZ

7795 Identification of Annexin A1 protein expression in human gastric adenocarcinoma using proteomics and tissue microarray
Zhang ZQ, Li XJ, Liu GT, Zhang XY, Xia Y, Wen H

META-ANALYSIS

7804 Is Dor fundoplication optimum after laparoscopic Heller myotomy for achalasia? A meta-analysis
Wei MT, He YZ, Deng XB, Zhang YC, Yang TH, Jin CW, Hu B, Wang ZQ

CASE REPORT

7813 Mesenteric vein thrombosis in a patient heterozygous for factor Ⅴ Leiden and G20210A prothrombin genotypes
Karmacharya P, Aryal MR, Donato A

7816 Development of enterohepatic fistula after embolization in ileal gastrointestinal stromal tumor: A case report
Lee YH, Koo JS, Jung CH, Chung SY, Lee JJ, Kim SY, Hyun JJ, Jung SW, Choung RS, Lee SW, Choi JH

7820 Unusual early-stage pancreatic sarcomatoid carcinoma
Ren CL, Jin P, Han CX, Xiao Q, Wang DR, Shi L, Wang DX, Chen H

World Journal of Gastroenterology
Volume 19 Number 43 November 21, 2013

Contents

APPENDIX	I-VI	Instructions to authors
ABOUT COVER		Editorial Board Member of *World Journal of Gastroenterology*, Jamal A Ibdah, MD, PhD, Professor, Director, Division of Gastroenterology and Hepatology, University of Missouri-Columbia, 319 jesse hall, Columbia, MO 65212, United States
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 1352 experts in gastroenterology and hepatology from 64 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).
FLYLEAF	I-IX	Editorial Board

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
November 21, 2013

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.v19.i43.7489

World J Gastroenterol 2013 November 21; 19(43): 7489-7493
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

EDITORIAL

Challenges in diagnosing adhesive small bowel obstruction

Thijs R van Oudheusden, Bart AC Aerts, Ignace HJT de Hingh, Misha DP Luyer

Thijs R van Oudheusden, Ignace HJT de Hingh, Misha DP Luyer, Department of Surgery, Catharina Hospital, 5623 EJ, Eindhoven, The Netherlands
Bart AC Aerts, Department of Surgery, Amphia Hospital, 4818 CK, Breda, The Netherlands
Author contributions: van Oudheusden TR, Aerts BAC wrote the editorial; de Hingh IHJT reviewed the editorial for intellectual content; Luyer MDP supervised and reviewed the editorial.
Correspondence to: Misha DP Luyer, MD, PhD, Department of Surgery, Catharina Hospital, Michelangelolaan 2, 5623 EJ, Eindhoven, The Netherlands. misha.luyer@catharinaziekenhuis.nl
Telephone: +31-40-2399111 Fax: +31-40-2455035
Received: July 16, 2013 Revised: September 15, 2013
Accepted: September 16, 2013
Published online: November 21, 2013

Abstract

Adhesive small bowel obstruction (ASBO) is the most frequently encountered surgical disorder of the small intestine. Up to 80% of ASBO cases resolve spontaneously and do not require invasive treatment. It is important to identify such patients that will benefit from conservative treatment in order to prevent unnecessarily exposing them to the risks associated with surgical intervention, such as morbidity and further adhesion formation. For the remaining ASBO patients, timely surgical intervention is necessary to prevent small bowel strangulation, which may cause intestinal ischemia and bowel necrosis. While early identification of these patients is key to decreasing ASBO-related morbidity and mortality, the non-specific signs and laboratory findings upon clinic presentation limit timely diagnosis and implementation of appropriate clinical management. Combining the clinical presentation findings with those from other diagnostic imaging modalities, such as abdominal X-ray, computed tomography-scan and water-soluble contrast studies, will improve diagnosis of ASBO and help clinicians to better evaluate the potential of conservative management as a safe strategy for a particular patient. Nonetheless, patients who present with moderate findings by all these approaches continue to represent a challenge. A new diagnostic strategy is urgently needed to further improve our ability to identify early signs of strangulated bowel, and this diagnostic modality should be able to indicate when surgical management is required. A number of potential serum markers have been proposed for this purpose, including intestinal fatty acid binding protein and α-glutathione S transferase. On-going research is attempting to clearly define their diagnostic utility and to optimize their potential role in determining which patients should be managed surgically.

Key words: Adhesive small bowel obstruction; Diagnosis; Clinical management; Biological markers; Intestinal fatty acid binding protein; α-glutathione S transferase

Core tip: Adhesive small bowel obstruction (ASBO) is a frequently encountered disorder of the small intestine following abdominal surgery. Accurately predicting whether ASBO patients can be treated conservatively is required to prevent exposing patients unnecessarily to surgery-related risks, including morbidity and further adhesion formation. Although recent technological developments have improved the ability to identify those patients most fit for conservative management, the remaining patients with moderate findings upon clinical presentation remain a problem. Serum markers of intestinal ischemia are promising candidates for improving early diagnosis and identification of patients with strangulated bowel, who will benefit most from surgical management.

van Oudheusden TR, Aerts BAC, de Hingh IHJT, Luyer MDP. Challenges in diagnosing adhesive small bowel obstruction. *World J Gastroenterol* 2013; 19(43): 7489-7493 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/

i43/7489.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7489

INTRODUCTION

Small bowel obstruction (SBO) leading to strangulation and potential bowel necrosis is a serious condition that mandates surgical intervention[1-3]. Timely diagnosis is essential to prevent the associated morbidity and mortality that manifest as operative management is delayed[4]. This fact was highlighted by the adage among surgeons citing "never let the sun rise or set in the case of small bowel obstruction".

Since up to 80% of SBO cases resolve without incident under conservative treatment[5,6], identification of patients whose obstruction will spontaneously resolve is important to prevent unnecessary surgical intervention and exposure to the risks of procedure-related morbidity and further formation of adhesions[6,7]. Recent technological advances in diagnostic modalities have improved the ability to identify patients who are most likely to benefit from conservative treatment; however, accurate and early identification of those patients who will ultimately require surgical intervention remains a challenge, especially when the clinical symptoms are moderate[2].

BACKGROUND

SBO is the most frequently encountered surgery-related disorder of the small intestine. In up to two-third of SBO cases, adhesions from prior abdominal surgery are implicated as the direct cause, having manifested as adhesive small bowel obstruction (ASBO)[2,6,8]. Although the majority of ASBO present within one year after surgery, up to 21% can develop up to ten years later[9]. In addition, other causes of SBOs exist, including neoplasma, herniations, inflammatory disease, or congenital disorders. Regardless of the cause, however, obstructed bowel eventually becomes edemateous, which leads to bowel ischemia, inflammation, and necrosis in the end-stage and requires surgical resection.

Clinical presentation

Patients with SBO usually present with a wide range of complaints, such as nausea, vomiting, and intermittent abdominal pain. In most cases, a history of prior abdominal surgery is present[6,10]. However, the clinical symptoms only contribute partially to diagnosis of ASBO, and studies have calculated the symptom-related sensitivity and specificity of acute abdominal pain as 75% and 99%[11]. One of the more recent studies, evaluating the current diagnostic technologies and clinical routines, found a higher overall sensitivity (88%) but a lower specificity (41%) for this parameter[12]; thus, improved diagnostic modalities are still needed. Unfortunately, the clinical symptoms of SBO are also not reliable predictors of the optimal disease management strategy, and distinguishing patients with bowel strangulation who require prompt surgical intervention remains a particular challenge to clinicians.

Laboratory findings

Laboratory tests are often used to confirm clinical suspicions and evaluate the degree of illness. The commonly measured inflammatory markers, such as white blood cell (WBC) count and C-reactive protein (CRP)[13], however, cannot distinguish inflammation due to obstruction from other inflammatory syndromes and are therefore of little value in the early diagnosis of ASBO[14]. Even in the case of bowel ischemia, as would be seen in bowel strangulation, studies have detected no significant differences in the WBC or CRP levels of patients who benefit from conservative management and those who require surgical intervention, making these markers useless for distinguishing these two categories of patients[14-16].

When progression to ischemia occurs, L-lactate, lactate dehydrogenase (LDH) and creatine kinase (CK) can rise due to hypoperfusion of the intestinal tissue[15]. However, large quantities of L-lactate are cleared by the liver during splanchnic hypoperfusion, resulting in L-lactate being increased at a very late stage of the process, when extensive intestinal infarction is already well established[17]. From a clinical perspective, a rise in L-lactate level increases sensitivity for detecting bowel ischemia up to 100% and is considered a strong indicator for emergency surgical intervention[18]. In contrast, LDH and CK levels rise in any ischemic condition, and are therefore unspecific. D-dimer, however, may serve as an exclusionary indicator for the presence of ischemia, due to its role as an enzymatic degradation product of fibrin, but it also lacks specificity since it can be elevated in numerous other conditions[14].

Since the above-mentioned markers are not specific enough for diagnosis of SBO they are also not useful for determining whether surgical intervention is needed for any particular case. Instead, these markers can be used to simply reflect severity of the disease and may contribute "circumstantial evidence" to support or deny a decision based upon a wide array of clinical findings.

Imaging techniques

The 2010 Bologna Guidelines for Diagnosis and Management of ASBO arose from an international consensus statement. According to these guidelines, all suspected cases of ASBO should be evaluated by abdominal X-ray (level 2b)[7]. Specifically, the presence or absence of classical signs, such as distension, > 3 cm diameter dilatation of the small bowel, perturbed air-fluid levels and absence of colonic gas, is considered a sufficient means of diagnosis, and studies have calculated this approach to have overall sensitivity and specificity ranging from 60%-85%[6,7].

In contrast, Laméris *et al*[12] showed that evaluating patients presenting with acute abdominal pain with plain radiography provided no benefit towards improving the above-mentioned sensitivity and specificity, presuming

that there is no role in the diagnostic work-up. Adding ultrasonography (US) after clinical diagnosis, however, was shown to increase the specificity from 41% to 85%. In suspected SBO cases, US can differentiate between ileus and mechanical obstruction, since peristalsis can be observed by this imaging modality[19]. Extra-luminal fluid findings are of major clinical importance as they are commonly used to make clinical decisions as to which surgical approach will be most tolerable and beneficial to a particular patient[20]. In contrast to these findings, the Bologna Guidelines state that there is limited value for US (level 2c), since entrapment of air in ASBO limits ultrasound transmission, making it a useful diagnostic tool only when applied by technical experts[2,7].

Using computed tomography (CT)-scan as an additional imaging platform to evaluate all patients with inconclusive plain radiologic films has proven highly useful for diagnosing SBO[2,7,21,22]. CT-scan has high sensitivity and specificity for SBO (> 92% and 93% respectively); in addition, the additional information provided by CT scanning can help to detect signs of intestinal ischemia or perforation[6,23-25]. However, Maglinte *et al*[26] reported that CT-scan can be just as sensitive as a plain abdominal x-ray for differentiating between obstruction and non-obstruction (86% *vs* 82% detection levels). It is important to note that the group with possible signs of ischemia remains a clinical challenge, and making a decision for clinical management is still a problem[10,23,27,28].

Magnetic resonance imaging (MRI) seems to have a limited role in diagnosing ASBO. MRI provides similar sensitivity and specificity as CT-scan, but no current guidelines have been established or implemented for applying MRI in standard clinical practice[2,7,29]. Interestingly, when combining abdominal films with water-soluble contrast medium, the approach can both make a diagnosis and safely rule-out the presence of a complete obstruction. In this manner, patient evaluation by water-soluble contrast studies can help to predict whether their ASBO can be treated conservatively or will require surgical intervention[7,10,22,30]. Besides being a useful diagnostic tool, water-soluble contrast may also have therapeutic potential; its ability to draw fluid into the lumen reduces edema in the gut wall, thereby relieving the obstruction and stimulating peristalsis[31]. A randomized controlled trial by Burge *et al*[5] showed an appreciable therapeutic effect when gastrografin was applied as the contrast agent to evaluate ASBO patients; specifically, a significantly accelerated resolution of the obstruction was seen in up to 75% of the patients within 24 h after the application. This result may be attributed to the hyperosmolar quality of gastrografin or other contrast mediums. While the precise benefit of contrast mediums reducing the need for surgery have yet to be systematically proven[30,32,33], their relation to reduced length of hospital stay has been demonstrated in several trials[5,28,31,32]. Certainly, however, those ASBO patients who show no contrast being able to enter the colon will require surgical treatment.

NEW DEVELOPMENTS

The limitations of the above-mentioned diagnostic modalities are likely to cause a delay in diagnosis. In recent years, several serum markers with potential to detect ischemic small bowel have been identified[13,14]. These markers include factors that are released by damaged enterocytes, such as intestinal fatty acid binding protein (I-FABP) and α-glutathione S transferase (α-GST). Enterocytes are rapidly shed in the early phases of intestinal injury and can be readily detected in both urine and plasma, providing promising possibilities for their use as early detection markers[33].

Plasma levels of the cytosolic protein α-GST rise in conjunction with ischemic intestinal damage; yet, this protein provides variable results as a diagnostic tool, with reported sensitivity ranging from 20%-100% and pooled specificity of 85%[14,15,34]. Therefore, α-GST may be more useful as an exclusion criterion, rather then as an indicator for surgical intervention. The other marker I-FABP, a cytosolic protein found in tissues involved in uptake and consumption of fatty acids, is released immediately by damaged small bowel, making it a very specific marker[35]. Patients presenting with SBO but without ischemia show normal levels of serum or urine I-FABP[36]. A recent clinical trial of patients with acute abdominal pain demonstrated that serum I-FABP levels were significantly higher in those patients with small bowel ischemia than in either those with non-ischemic small bowel disease or those without small bowel disease[16]. Furthermore, a majority (57.7%) of these ischemic patients had strangulated bowel. Thus, I-FABP may have a role in selecting candidates for surgical intervention. Other putative candidate markers are D-lactate and claudin[15,37,38]; however, the low specificity of D-lactate and lack of substantial evidence for a role of claudin 3 in SBO makes it difficult to clearly define their potential.

Besides these plasma markers, the prediction model developed by Komatsu *et al*[39] has identified older age, presence of ascites, and high-volume nasogastral tube drainage on day 3 as critical factors in patients who initially received conservative treatment. Unfortunately, this study did not include findings from radiographic imaging or oral water-soluble studies in the analysis. Although the prediction model is promising, it is necessary to consider the potential impact of markers specifically released by the obstructed small bowel in an earlier stage.

CONCLUSION

Despite the remarkable technological advances in diagnosis of ASBO, the challenge of determining how to most effectively and safely manage these cases remains. Our ability to identify patients who can be treated conservatively has improved greatly, but the same has not been achieved for patients who will require emergency surgery, especially when their presenting symptoms are moderate. Serum markers have emerged as promising

candidates for early diagnosis of strangulated bowel, but further research is necessary to clarify their clinical value in the disease management.

REFERENCES

1 **Arung W**, Meurisse M, Detry O. Pathophysiology and prevention of postoperative peritoneal adhesions. *World J Gastroenterol* 2011; **17**: 4545-4553 [PMID: 22147959 DOI: 10.3748/wjg.v17.i41.4545]

2 **Diaz JJ**, Bokhari F, Mowery NT, Acosta JA, Block EF, Bromberg WJ, Collier BR, Cullinane DC, Dwyer KM, Griffen MM, Mayberry JC, Jerome R. Guidelines for management of small bowel obstruction. *J Trauma* 2008; **64**: 1651-1664 [PMID: 18545135 DOI: 10.1097/TA.0b013e31816f709e]

3 **Takeuchi K**, Tsuzuki Y, Ando T, Sekihara M, Hara T, Yoshikawa M, Kuwano H. Clinical studies of strangulating small bowel obstruction. *Am Surg* 2004; **70**: 40-44 [PMID: 14964545]

4 **Fevang BT**, Fevang JM, Søreide O, Svanes K, Viste A. Delay in operative treatment among patients with small bowel obstruction. *Scand J Surg* 2003; **92**: 131-137 [PMID: 12841553]

5 **Burge J**, Abbas SM, Roadley G, Donald J, Connolly A, Bissett IP, Hill AG. Randomized controlled trial of Gastrografin in adhesive small bowel obstruction. *ANZ J Surg* 2005; **75**: 672-674 [PMID: 16076330 DOI: 10.1111/j.1445-2197.2005.03491.x]

6 **Kendrick ML**. Partial small bowel obstruction: clinical issues and recent technical advances. *Abdom Imaging* 2009; **34**: 329-334 [PMID: 18597140 DOI: 10.1007/s00261-008-9436-0]

7 **Catena F**, Di Saverio S, Kelly MD, Biffl WL, Ansaloni L, Mandalà V, Velmahos GC, Sartelli M, Tugnoli G, Lupo M, Mandalà S, Pinna AD, Sugarbaker PH, Van Goor H, Moore EE, Jeekel J. Bologna Guidelines for Diagnosis and Management of Adhesive Small Bowel Obstruction (ASBO): 2010 Evidence-Based Guidelines of the World Society of Emergency Surgery. *World J Emerg Surg* 2011; **6**: 5 [PMID: 21255429 DOI: 10.1186/1749-7922-6-5]

8 **Attard JA**, MacLean AR. Adhesive small bowel obstruction: epidemiology, biology and prevention. *Can J Surg* 2007; **50**: 291-300 [PMID: 17897517]

9 **Menzies D**, Ellis H. Intestinal obstruction from adhesions--how big is the problem? *Ann R Coll Surg Engl* 1990; **72**: 60-63 [PMID: 2301905]

10 **Wilson MS**, Ellis H, Menzies D, Moran BJ, Parker MC, Thompson JN. A review of the management of small bowel obstruction. Members of the Surgical and Clinical Adhesions Research Study (SCAR). *Ann R Coll Surg Engl* 1999; **81**: 320-328 [PMID: 10645174]

11 **Eskelinen M**, Ikonen J, Lipponen P. Contributions of history-taking, physical examination, and computer assistance to diagnosis of acute small-bowel obstruction. A prospective study of 1333 patients with acute abdominal pain. *Scand J Gastroenterol* 1994; **29**: 715-721 [PMID: 7973431 DOI: 10.3109/00365529409092499]

12 **Laméris W**, van Randen A, van Es HW, van Heesewijk JP, van Ramshorst B, Bouma WH, ten Hove W, van Leeuwen MS, van Keulen EM, Dijkgraaf MG, Bossuyt PM, Boermeester MA, Stoker J. Imaging strategies for detection of urgent conditions in patients with acute abdominal pain: diagnostic accuracy study. *BMJ* 2009; **338**: b2431 [PMID: 19561056 DOI: 10.1136/bmj.b2431]

13 **Derikx JP**, Luyer MD, Heineman E, Buurman WA. Non-invasive markers of gut wall integrity in health and disease. *World J Gastroenterol* 2010; **16**: 5272-5279 [PMID: 21072889 DOI: 10.3748/wjg.v16.i43.5272]

14 **Block T**, Nilsson TK, Björck M, Acosta S. Diagnostic accuracy of plasma biomarkers for intestinal ischaemia. *Scand J Clin Lab Invest* 2008; **68**: 242-248 [PMID: 17934974 DOI: 10.1080/00365510701646264]

15 **Evennett NJ**, Petrov MS, Mittal A, Windsor JA. Systematic review and pooled estimates for the diagnostic accuracy of serological markers for intestinal ischemia. *World J Surg* 2009; **33**: 1374-1383 [PMID: 19424744 DOI: 10.1007/s00268-009-0074-7]

16 **Kanda T**, Tsukahara A, Ueki K, Sakai Y, Tani T, Nishimura A, Yamazaki T, Tamiya Y, Tada T, Hirota M, Hasegawa J, Funaoka H, Fujii H, Hatakeyama K. Diagnosis of ischemic small bowel disease by measurement of serum intestinal fatty acid-binding protein in patients with acute abdomen: a multicenter, observer-blinded validation study. *J Gastroenterol* 2011; **46**: 492-500 [PMID: 21298292 DOI: 10.1007/s00535-011-0373-2]

17 **Jakob SM**, Merasto-Minkkinen M, Tenhunen JJ, Heino A, Alhava E, Takala J. Prevention of systemic hyperlactatemia during splanchnic ischemia. *Shock* 2000; **14**: 123-127 [PMID: 10947154 DOI: 10.1097/00024382-200014020-00008]

18 **Lange H**, Jäckel R. Usefulness of plasma lactate concentration in the diagnosis of acute abdominal disease. *Eur J Surg* 1994; **160**: 381-384 [PMID: 7948358]

19 **Grassi R**, Romano S, D'Amario F, Giorgio Rossi A, Romano L, Pinto F, Di Mizio R. The relevance of free fluid between intestinal loops detected by sonography in the clinical assessment of small bowel obstruction in adults. *Eur J Radiol* 2004; **50**: 5-14 [PMID: 15093230 DOI: 10.1016/j.ejrad.2003.11.009]

20 **Di Mizio R**, Grassi R, Marchese E, Basti M, Di Campli G, Catalano O, Rotondo A, Fanucci A. ["Uncompensated" small bowel obstruction in adults. Ultrasonographic findings of free fluid between loops and its prognostic value]. *Radiol Med* 1995; **89**: 787-791 [PMID: 7644729]

21 **Obuz F**, Terzi C, Sökmen S, Yilmaz E, Yildiz D, Füzün M. The efficacy of helical CT in the diagnosis of small bowel obstruction. *Eur J Radiol* 2003; **48**: 299-304 [PMID: 14652150]

22 **Trésallet C**, Lebreton N, Royer B, Leyre P, Godiris-Petit G, Menegaux F. Improving the management of acute adhesive small bowel obstruction with CT-scan and water-soluble contrast medium: a prospective study. *Dis Colon Rectum* 2009; **52**: 1869-1876 [PMID: 19966635 DOI: 10.1007/DCR.0b013e3181b35c06]

23 **Mallo RD**, Salem L, Lalani T, Flum DR. Computed tomography diagnosis of ischemia and complete obstruction in small bowel obstruction: a systematic review. *J Gastrointest Surg* 2005; **9**: 690-694 [PMID: 15862265]

24 **Zalcman M**, Sy M, Donckier V, Closset J, Gansbeke DV. Helical CT signs in the diagnosis of intestinal ischemia in small-bowel obstruction. *AJR Am J Roentgenol* 2000; **175**: 1601-1607 [PMID: 11090385 DOI: 10.2214/ajr.175.6.1751601]

25 **Zielinski MD**, Eiken PW, Bannon MP, Heller SF, Lohse CM, Huebner M, Sarr MG. Small bowel obstruction-who needs an operation? A multivariate prediction model. *World J Surg* 2010; **34**: 910-919 [PMID: 20217412 DOI: 10.1007/s00268-010-0479-3]

26 **Maglinte DD**, Reyes BL, Harmon BH, Kelvin FM, Turner WW, Hage JE, Ng AC, Chua GT, Gage SN. Reliability and role of plain film radiography and CT in the diagnosis of small-bowel obstruction. *AJR Am J Roentgenol* 1996; **167**: 1451-1455 [PMID: 8956576 DOI: 10.2214/ajr.167.6.8956576]

27 **Jancelewicz T**, Vu LT, Shawo AE, Yeh B, Gasper WJ, Harris HW. Predicting strangulated small bowel obstruction: an old problem revisited. *J Gastrointest Surg* 2009; **13**: 93-99 [PMID: 18685902 DOI: 10.1007/s11605-008-0610-z]

28 **Wadani HA**, Al Awad NI, Hassan KA, Zakaria HM, Alaqeel FO. Role of water soluble contrast agents in assigning patients to a non-operative course in adhesive small bowel obstruction. *Oman Med J* 2011; **26**: 454-456 [PMID: 22253960 DOI: 10.5001/omj.2011.116]

29 **Fidler J**. MR imaging of the small bowel. *Radiol Clin North Am* 2007; **45**: 317-331 [PMID: 17502220 DOI: 10.1016/j.rcl.2007.03.012]

30 **Branco BC**, Barmparas G, Schnüriger B, Inaba K, Chan LS, Demetriades D. Systematic review and meta-analysis of the

diagnostic and therapeutic role of water-soluble contrast agent in adhesive small bowel obstruction. *Br J Surg* 2010; **97**: 470-478 [PMID: 20205228 DOI: 10.1002/bjs.7019]

31 **Di Saverio S**, Catena F, Ansaloni L, Gavioli M, Valentino M, Pinna AD. Water-soluble contrast medium (gastrografin) value in adhesive small intestine obstruction (ASIO): a prospective, randomized, controlled, clinical trial. *World J Surg* 2008; **32**: 2293-2304 [PMID: 18688562 DOI: 10.1007/s00268-008-9694-6]

32 **Abbas SM**, Bissett IP, Parry BR. Meta-analysis of oral water-soluble contrast agent in the management of adhesive small bowel obstruction. *Br J Surg* 2007; **94**: 404-411 [PMID: 17380561 DOI: 10.1002/bjs.5775]

33 **Derikx JP**, Matthijsen RA, de Bruïne AP, van Bijnen AA, Heineman E, van Dam RM, Dejong CH, Buurman WA. Rapid reversal of human intestinal ischemia-reperfusion induced damage by shedding of injured enterocytes and reepithelialisation. *PLoS One* 2008; **3**: e3428 [PMID: 18927609 DOI: 10.1371/journal.pone.0003428]

34 **Delaney CP**, O'Neill S, Manning F, Fitzpatrick JM, Gorey TF. Plasma concentrations of glutathione S-transferase isoenzyme are raised in patients with intestinal ischaemia. *Br J Surg* 1999; **86**: 1349-1353 [PMID: 10540149 DOI: 10.1046/j.1365-2168.1999.01245.x]

35 **Lieberman JM**, Sacchettini J, Marks C, Marks WH. Human intestinal fatty acid binding protein: report of an assay with studies in normal volunteers and intestinal ischemia. *Surgery* 1997; **121**: 335-342 [PMID: 9068676 DOI: 10.1016/S0039-6060(97)90363-9]

36 **Kanda T**, Fujii H, Tani T, Murakami H, Suda T, Sakai Y, Ono T, Hatakeyama K. Intestinal fatty acid-binding protein is a useful diagnostic marker for mesenteric infarction in humans. *Gastroenterology* 1996; **110**: 339-343 [PMID: 8566578 DOI: 10.1053/gast.1996.v110.pm8566578]

37 **Thuijls G**, Derikx JP, de Haan JJ, Grootjans J, de Bruïne A, Masclee AA, Heineman E, Buurman WA. Urine-based detection of intestinal tight junction loss. *J Clin Gastroenterol* 2010; **44**: e14-e19 [PMID: 19525861 DOI: 10.1097/MCG.0b013e31819f5652]

38 **van Noord D**, Mensink PB, de Knegt RJ, Ouwendijk M, Francke J, van Vuuren AJ, Hansen BE, Kuipers EJ. Serum markers and intestinal mucosal injury in chronic gastrointestinal ischemia. *Dig Dis Sci* 2011; **56**: 506-512 [PMID: 20628816 DOI: 10.1007/s10620-010-1303-5]

39 **Komatsu I**, Tokuda Y, Shimada G, Jacobs JL, Onodera H. Development of a simple model for predicting need for surgery in patients who initially undergo conservative management for adhesive small bowel obstruction. *Am J Surg* 2010; **200**: 215-223 [PMID: 20591400 DOI: 10.1016/j.amjsurg.2009.07.045]

P- Reviewer: Buchs NC **S- Editor:** Wen LL
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.v19.i43.7494

World J Gastroenterol 2013 November 21; 19(43): 7494-7499
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

High intensity focused ultrasound, liver disease and bridging therapy

Mearini Luigi

Mearini Luigi, Deparment of Urology, University of Perugia, Azienda Ospedaliera di Perugia, 06100 Perugia, Italy
Author contributions: Mearini L collected the materials and wrote the manuscript.
Correspondence to: Mearini Luigi, MD, Urologist-Oncologist, Deparment of Urology, University of Perugia, Azienda Ospedaliera di Perugia, Sant'Andrea delle Fratte, 06100 Perugia, Italy. luigi.mearini@tin.it
Telephone: +39-338-2116845 Fax: +39-75-5784416
Received: August 7, 2013 Revised: September 10, 2013
Accepted: September 16, 2013
Published online: November 21, 2013

Abstract

High-intensity focused ultrasound (HIFU) is a non-invasive modality that uses an extracorporeal source of focused ultrasound energy. This technique was introduced by Lynn *et al* and is able to induce coagulative necrosis in selected tissues without damaging adjacent structures. Although HIFU has been studied for 50 years, recent technological developments now allow its use for tumours of the liver, prostate and other sites. In liver disease, HIFU has been used to treat unresectable, advanced stages of hepatocellular carcinoma (HCC) and liver metastases. Hepatocellular carcinoma is a serious health problem worldwide and is endemic in some areas because of its association with hepatitis B and C viruses (in 20% of cases). Liver transplantation (LT) has become one of the best treatments available because it removes both the tumour and the underlying liver disease such as cirrhosis (which is present in approximately 80% of cases). The prerequisite for long-term transplant success depends on tumour load and strict selection criteria regarding the size and number of tumour nodules. The need to obtain the optimal benefit from the limited number of organs available has prompted strict selection criteria limited to only those patients with early HCC who have a better long-term outcome after LT. The so-called "bridging therapy" has the aim of controlling disease burden for patients who are on the organ transplant waiting list. Amongst various treatment options, transarterial chemoembolisation and radiofrequency ablation are the most popular treatment choices. Recently, Cheung *et al* demonstrated that HIFU ablation is a safe and effective method for the treatment of HCC patients with advanced cirrhosis as a bridging therapy and that it reduced the drop-out rate from the liver transplant waiting list. In this commentary, we discuss the current value of HIFU in the treatment of liver disease, including its value as a bridging therapy, and examine the potential advantages of other therapeutic strategies.

Key words: High-intensity focused ultrasound; Hepatocellular carcinoma; Liver transplantation; Bridging therapy; Waiting list

Core tip: High-intensity focused ultrasound (HIFU) is a non-invasive modality used to destroy tissue. It has been used to treat unresectable advanced stages of hepatocellular carcinoma (HCC) and liver metastases. In some HCC cases, liver transplantation has become one of the best treatments because it removes the tumour and the underlying liver disease such as cirrhosis. The so-called "bridging therapy" has the aim of controlling disease burden for patients who are on the organ transplant waiting list. Here, we discuss various treatment options including transarterial chemoembolisation and radiofrequency ablation, and we examine the utility of HIFU as a safe and effective method of bridging therapy that can reduce the dropout rate of patients who are on the liver transplant waiting list.

Luigi M. High intensity focused ultrasound, liver disease and bridging therapy. *World J Gastroenterol* 2013; 19(43): 7494-7499 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7494.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7494

COMMENTARY ON HOT TOPICS

I have read with great interest the recent article by Cheung *et al*[1] reporting their experience in the use of high-intensity focused ultrasound (HIFU) in patients with hepatocellular carcinoma (HCC) and cirrhosis who are waiting for liver transplant. The study aim was to determine whether HIFU could reduce the patient dropout rate.

HIFU is a non-invasive modality that uses an extracorporeal source of focused ultrasound energy. The technique was introduced by Lynn *et al*[2], and it is able to induce coagulative necrosis in targeted tissues without damaging overlying and surrounding vital structures.

Although HIFU has been studied for 50 years, recent technological developments have allowed its use in treating tumours of the liver[3], prostate[4] and other sites[5].

HIFU is a highly precise medical procedure that applies high-intensity focused energy to "heat" and "destroy" diseased tissues. Its precision is under investigation for a possible application as "focal" therapy in case of prostate cancer, where whole gland therapy has a negative impact in terms of incontinence and impotence[6].

HIFU is a hyperthermia therapy, which is a class of clinical therapies [including radiofrequency ablation (RFA)] that use high temperature to treat diseases.

HIFU is also a modality of therapeutic ultrasound, involving minimally invasive or non-invasive methods to direct acoustic energy into the body. In addition to HIFU, other modalities include ultrasound-assisted drug delivery, ultrasound haemostasis, ultrasound lithotripsy and ultrasound-assisted thrombolysis.

A clinical HIFU procedure is typically performed in conjunction with an imaging procedure to enable treatment planning and targeting before applying any therapeutic or ablative levels of ultrasound energy. When diagnostic sonography is used, the technique is termed ultrasound-guided focused ultrasound (USgHIFU or USgFUS). Magnetic resonance imaging is also used for guidance; thus, the technique is sometimes called magnetic resonance-guided focused ultrasound, which is often shortened to MRgHIFU or MRgFUS.

Currently, USgHIFU is approved for use in Bulgaria, China, Hong Kong, Italy, Japan, Korea, Malaysia, Mexico, Russia, Romania, Spain and the United Kingdom. MRgHIFU is an approved therapeutic procedure to treat uterine fibroids in Asia, Australia, Canada, Europe, Israel and the United States (Food and Drug Administration, FDA approved). Research on other indications is actively underway, including clinical trials evaluating the effectiveness of HIFU for the treatment of cancers of the brain, breast, liver, bone, and prostate.

From a technical point of view, the ultrasound waves of high-intensity focused ultrasound are generated by high frequency (0.5 to 10 MHz) vibration of a piezoelectric or piezo-ceramic transducer. The ultrasound beams are then focused by spherical arrangement using an acoustic lens or parabolic reflectors into a small, discrete region that corresponds to the focal point. For clinical applications, and similar to ultrasound imaging, an ultrasound probe is usually coupled by degassed water between the source and patient surface (skin, rectal wall). Because of the comparable acoustic properties of water and tissue, the sound waves should penetrate the surface and the pre-target tissue with only slight absorption, reflection and heating. This phenomenon occurs because the power density of the converging ultrasound increases as it approaches the focal point. The focal region is a 3-dimensional zone, whose area depends on the frequency and the geometry of the source. Generally, the focal area is approximately 10 to 50 mm in length and 1 to 5 mm in diameter.

Based on target volume, the tissue can be ablated by sequentially shifting the focal zone with incremental movements of the transducer. This approach is combined with adjustments of the focal length and is coupled with an immobile organ or with the complex real-time tracking of a moving target (such as liver). The extent of tissue ablation is approximately that of the physical focal zone, although in practice cold spots (cause by blood perfusion in the tissue), beam distortion and beam misregistration are impediments to finely controlled treatments. However, by scanning the target using multiple pulses and multiple focal points, large tissue areas can be ablated.

The effect of acoustic cavitation induced by the ultrasound beam is complex, and acoustic impedance is sometimes unpredictable. However, the result is cell necrosis induced through a combination of mechanical stress and thermal injury.

The mechanical effect is induced by cavitation, a process in which bubbles develop and increase in size to the point at which resonance is achieved. The bubble formation is a consequence of the negative pressure of the ultrasound wave. As the bubbles expand and collapse, high pressures ranging from 20000 to 30000 bars develop and damage nearby cells. The popcorn effect is the typical example of cavitation.

The thermal effect is directly induced by the ultrasound beams, and due to the significant energy deposition at the focus the temperature within the tissue can rise from 65 to 85 ℃. The temperature increase destroys tissue by coagulative necrosis. Higher temperatures are typically avoided to prevent boiling of liquids inside the tissue.

Because ultrasound destroys the diseased tissue non-invasively, it is also known as a non-invasive surgery. In liver disease, HIFU has been used for the treatment of unresectable, advanced stages of hepatocellular carcinoma or for the treatment of liver metastases.

Previous studies have shown that HIFU is safe and effective for patients with hepatocellular carcinoma[7] and can improve the quality of life of patients with HCC. In a study involving 145 patients with HCC, symptoms improved or pain was relieved in 84.8% of the 145 patients. Additionally, the size of the target tumour shrank by various degrees. The 2-year survival rate was 80% in patients with stage Ⅰb HCC, 51.4% in stage Ⅱa, and 46.5% in stage Ⅲa.

Ng *et al*[8] involving 49 patients receiving HIFU for unresectable HCC showed that the technique was effective in 79.5% of cases. The study found that only tumour size (≥ 3.0 cm) was a significant risk factor affecting the complete ablation rate. The 1- and 3-year overall survival rates were 87.7% and 62.4%, respectively. Moreover, HIFU is safe for the treatment of disease adjacent to or surrounding a major liver vessel. The study by Zhang *et al*[9] enrolled 39 patients with HCC. All of the treated tumours had a distance between the tumour and main blood vessel (inferior vena cava, main hepatic vein branches, portal vein) of less than 1 cm, and no major blood vessel injury was observed in any subject.

HIFU has been used in combination with transarterial chemoembolisation (TACE) in prior studies. Jin *et al*[10] reported their experience of HIFU and transraterial chemoembolisation in 73 patients with unresectable HCC. That study demonstrated that 45.2% patients achieved complete tumour ablation. By multivariate analysis, ablation response ($P = 0.001$) and tumour size ($P = 0.013$) were major prognostic factors in predicting response to therapy. In an interesting randomised trial comparing TACE alone *vs* TACE + HIFU, Li *et al*[11] showed that the total effective rate for tumour response was 72.8% in the TACE + HIFU group. This response was significantly higher than in the TACE group alone (44.5%, $P < 0.05$). The corresponding 1-, 2-, 3- and 5-year overall survival rates for the TACE-HIFU group were 72.7%, 50.0%, 31.8% and 11.4%, respectively. These rates were higher than in the TACE alone group (47.2%, 16.7%, 2.8% and 0%, respectively , $P < 0.01$).

HIFU ablation is well tolerated in HCC patients with cirrhosis. According to Cheung *et al*[12], 13% of 100 patients developed 18 complications. Morbidity was mainly caused by skin and subcutaneous tissue injuries in nine cases. Based on the Clavien classification of surgical complications, only four complications were grade 3a, while the other 14 were below this grade. By univariate analysis, only age was found to be an independent factor for poor HIFU tolerance.

HCC is a serious health problem worldwide because of its association with hepatitis B and C viruses. Liver transplantation has become one of the best HCC treatments available because it removes both the tumour and the underlying liver disease.

A prerequisite for the long-term success of a transplantation program depends on tumour load and selection criteria regarding size and number of tumour nodules. The need to obtain the optimal benefit from the limited number of available organs has prompted the use of careful selection criteria to list only those patients with early HCC who have a prediction of superior long-term outcome after LT.

Patients who fulfil the so-called Milan criteria (single tumour ≤ 5 cm; two or three tumours, none > 3 cm; no vascular invasion) or the expanded University of California San Francisco criteria (UCSF criteria: single tumour ≤ 4.5 cm; two or three tumours, none > 4.5 cm; or total tumour diameter ≤ 8 cm; no vascular invasion) may have a 3-year survival of up to 88%. However, the expansion of these criteria for transplantation is still a topic of discussion.

Other problems arise from the differential between the number of patients on the liver transplant waiting list and the number of available donors. Additionally, there is a time lag between patient inclusion on a waiting list and the available organ. For example, in the United States, more than 2000 candidates die each year while awaiting transplantation.

Some therapies for HCC, called "bridge therapy", have the capacity to "fix" or suspend tumour progression and to allow HCC patients to maintain active candidacy as long as necessary to obtain a liver. Several techniques are employed as bridge therapies for HCC patients awaiting liver transplantation. Treatment options such as TACE and radio frequency ablasion (RFA) are the most popular treatment choices as pre-transplant locoregional therapy.

Moreover, other goals of locoregional therapy, *e.g.*, alcohol injection, radiofrequency ablation, transarterial chemoembolisation, transarterial radioembolization, and liver resection, are intended to decrease tumour size and number in patients who initially present with tumours that do not meet locally acceptable criteria for liver transplantation.

The TACE principle is intra-arterial injection of cytotoxic drug combinations (doxorubicin and/or cisplatin and/or mitomycin into the hepatic artery), followed by lipiodol injection, gelfoam for vessel occlusion and degradable microspheres. An aggressive ablation therapy in association with a short transplant waiting time has the potential to optimise the curative intent of liver transplantation in selected cirrhotic patients. Based on the local extension of the disease and the hepatic functional reserve, TACE may be performed as a "complete", selective or superselective procedure through a microcatheter. Contraindications for TACE include Child-Pugh C liver cirrhosis, presence of multifocal bilobar tumour spread, presence of extrahepatic metastases, portal vein thrombosis or arterio-portal fistula.

TACE has shown excellent outcomes as a bridging therapy. However, only patients with preserved liver function and asymptomatic multinodular tumours without vascular invasion or extrahepatic spread are eligible for TACE because it avoids hepatic failure and severe adverse events[13]. TACE has been used as a selective/superselective procedure and has shown excellent results that are superior to a simple lobar approach[14]. As a bridging (or down-staging) therapy, selective/superselective TACE induces a histological necrosis in 91.8% of cases and was maximal for tumours > 3 cm.

RFA represents a widely applied method to treat HCC in a palliative intent, or as a "bridging" to liver transplantation. RFA may be performed under ultrasonography, Computed tomography guidance, or during laparoscopic and open surgical procedures. This procedure has more limitations than TACE, including the number of nodules

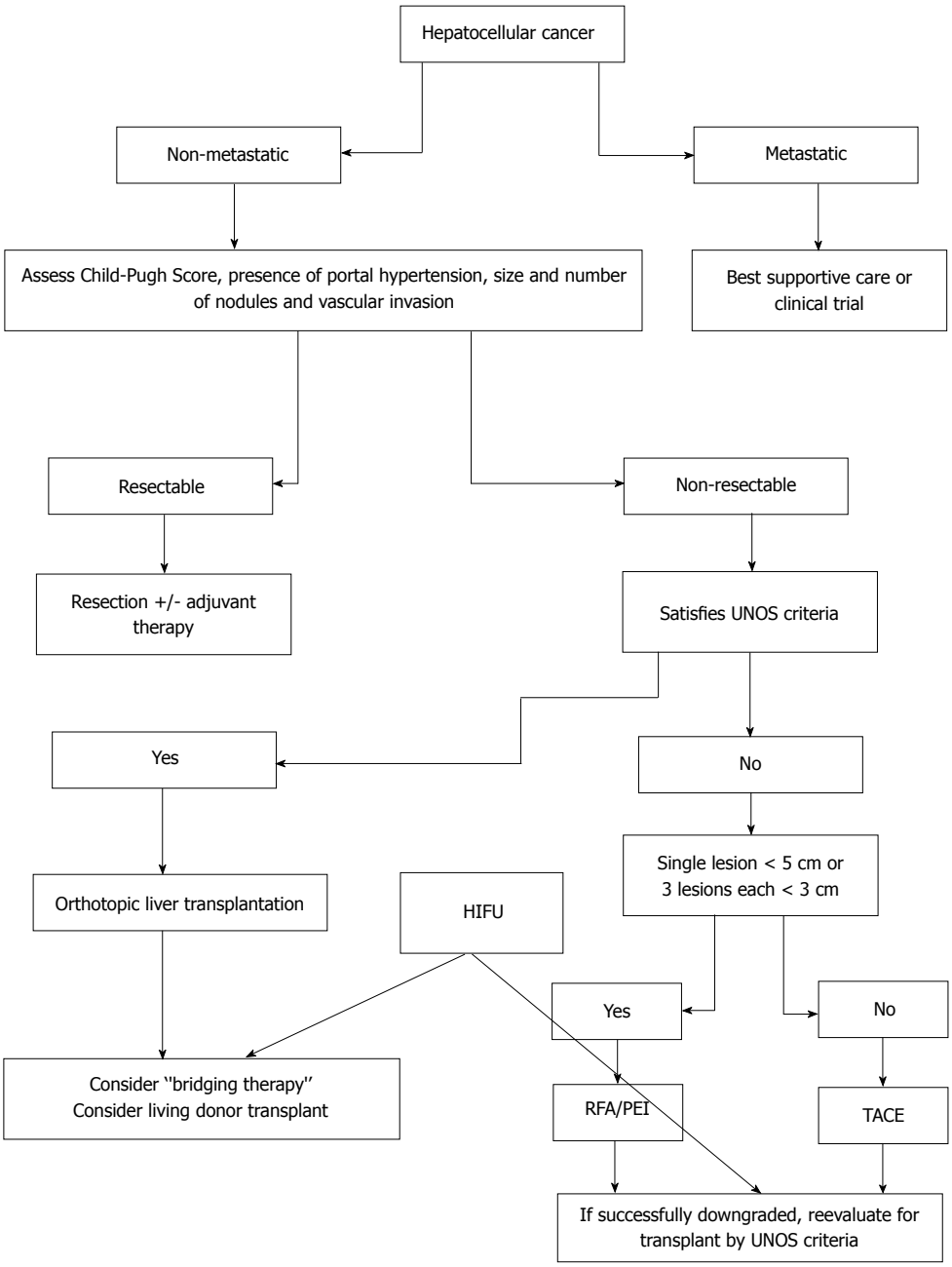


Figure 1 Approach to the management of newly diagnosed hepatocellular cancer. Source from Parikh *et al*[19]. "Bridging therapy": Surgical resection, radiofrequency ablation (RFA), transcatheter arterial chemoembolization (TACE). High-intensity focused ultrasound (HIFU) as proposal. PEI: Percutaneous ethanol injection; UNOS: United network for organ sharing.

that may be treated (up to three in most cases) or the maximal tumour diameter of the nodules (up to 5 cm). Effective treatment has been achieved[15] when 100% tumour necrosis is present. However, it is difficult to reach this goal with tumours exceeding the above-mentioned diameter or number of tumour nodules. Mazzaferro *et al*[16] showed that although the complete response rate was high (55%), tumour size (> 3 cm) and time from treatment (> 1 year) predict a high risk of tumour persistence in the targeted nodule.

As a bridging therapy, RFA showed some limitations. Schroeder *et al*[17] demonstrated that although the majority of treated patients (62%) had a solitary tumour at the beginning of treatment, tumour progression was observed in a large proportion (38%) of patients. These results limit the role of RFA as a bridging treatment prior to LT.

Yttrium-90 (Y90) microsphere radio-embolisation is a recently FDA-approved, non-surgical procedure used to treat inoperable HCC. This innovative procedure delivers targeted, internal radiation therapy directly to the tumour[18]. Some promising results have been reported for this technique either as a "bridging" option before other treatment modalities (partial hepatectomy, liver transplantation) or as a main therapy for patients with diffuse intrahepatic tumour spread. Treatment with Y90 microspheres has the advantage of being able to treat all intrahepatic

HCC lesions, including otherwise undetected tumours. This treatment may also be the alternative to TACE in selected patients with contraindications for TACE.

In conclusion, with increases in waiting times for liver transplantation, it has become common practice to monitor patients to ensure that they remain within the acceptable criteria for liver transplantation. Moreover, the dropout of patients on the waiting list is common because of cancer progression or other medical reasons. Locoregional therapy as a bridging strategy for patients on the waiting list aims to decrease tumour-related dropout rates and to reduce the incidence of recurrent diseases after liver transplantation. Current available techniques show a dropout rate up to 35% for transarterial embolisation and up to 15% for radiofrequency ablation.

Cheung *et al*[12] must be congratulated for testing the utility of high-intensity focused ultrasound in this particular setting. The study examined 49 consecutive HCC patients listed for liver transplant by UCSF criteria. Twenty-nine patients received TACE as a bridging therapy, 16 patients received no treatment before liver transplantation, and five patients received HIFU as bridging therapy. The control group of five patients received HIFU but were not on the transplant list. TACE was performed using cisplatin as the chemotherapeutic agent, and it was delivered with Lipiodol, followed by gelfoam particle embolisation. Selective cannulation and embolisation of the feeding arteries of the tumours were performed whenever possible. All of the HIFU treatments were conducted by an experienced hepatobiliary surgeon and radiologist using the JC HIFU system (Chongqing Haifu Technology, Chongqing, China). The system is composed of a real-time diagnostic imaging unit, a therapeutic unit, a degassed water circulation unit, and a computer system. The real-time diagnostic imaging unit provides direct visualisation of the tumour. The therapeutic unit consists of an ultrasound energy transducer that focuses the ultrasound energy at a 12-cm focal point. The degassed water circulation unit provides a medium for ultrasound transmission outside the body. The computer system controls these three units.

Cheung demonstrated that 90% of patients receiving HIFU had complete tumour response, while 10% had partial response. There was no complete or partial tumour response in the TACE group. Fourteen (46%) patients had progressive disease, and 14 (46%) patients had stable disease. The overall dropout rate was 24.1%.

HIFU was shown to be a safe treatment, and none of the patients receiving HIFU as a bridging therapy developed complications due to intolerance after the procedure. The complication rate was 8.2%, and the complications involved mild skin oedema and injury due to energy accumulation at the ultrasound beam pathway.

HIFU ablation is an entirely extracorporeal non-invasive ablative treatment method using focused ultrasound energy. It is capable of causing coagulative necrosis of the targeted HCC *via* intact skin, without the need for surgical incision.

HIFU has been well demonstrated to be an effective ablation modality that is non-invasive. It can effectively reduce the dropout rate from the liver transplant waiting list by providing effective tumour control. The histological proof from the liver explants provides evidence that the necrosis is effective in an *in vivo* model.

Despite the low number of enrolled subjects, the preliminary study by Cheung *et al*[1] is interesting and suggests the need for more extensive clinical trials that focus on the use of HIFU as a bridging therapy (Figure 1).

REFERENCES

1 **Cheung TT**, Fan ST, Chan SC, Chok KS, Chu FS, Jenkins CR, Lo RC, Fung JY, Chan AC, Sharr WW, Tsang SH, Dai WC, Poon RT, Lo CM. High-intensity focused ultrasound ablation: an effective bridging therapy for hepatocellular carcinoma patients. *World J Gastroenterol* 2013; **19**: 3083-3089 [PMID: 23716988 DOI: 10.3748/wjg.v19.i20.3083]

2 **Lynn JG**, Zwemer RL, Chick AJ, Miller AE. A new method for the generation and use of focused ultrasound in experimental biology. *J Gen Physiol* 1942; **26**: 179-193 [PMID: 19873337 DOI: 10.1085/jgp.26.2.179]

3 **Shen HP**, Gong JP, Zuo GQ. Role of high-intensity focused ultrasound in treatment of hepatocellular carcinoma. *Am Surg* 2011; **77**: 1496-1501 [PMID: 22196664]

4 **Mearini L**, Porena M. Transrectal high-intensity focused ultrasound for the treatment of prostate cancer: past, present, and future. *Indian J Urol* 2010; **26**: 4-11 [PMID: 20535278 DOI: 10.4103/0970-1591.60436]

5 **Kennedy JE**, Ter Haar GR, Cranston D. High intensity focused ultrasound: surgery of the future? *Br J Radiol* 2003; **76**: 590-599 [PMID: 14500272 DOI: 10.1259/bjr/17150274]

6 **Mearini L**, Porena M. Pros and cons of focal therapy for localised prostate cancer. *Prostate Cancer* 2011; **2011**: 584784 [PMID: 22110990 DOI: 10.1155/2011/584784]

7 **Xu G**, Luo G, He L, Li J, Shan H, Zhang R, Li Y, Gao X, Lin S, Wang G. Follow-up of high-intensity focused ultrasound treatment for patients with hepatocellular carcinoma. *Ultrasound Med Biol* 2011; **37**: 1993-1999 [PMID: 22036638 DOI: 10.1016/j.ultrasmedbio.2011.08.011]

8 **Ng KK**, Poon RT, Chan SC, Chok KS, Cheung TT, Tung H, Chu F, Tso WK, Yu WC, Lo CM, Fan ST. High-intensity focused ultrasound for hepatocellular carcinoma: a single-center experience. *Ann Surg* 2011; **253**: 981-987 [PMID: 21394012 DOI: 10.1097/SLA.0b013e3182128a8b]

9 **Zhang L**, Zhu H, Jin C, Zhou K, Li K, Su H, Chen W, Bai J, Wang Z. High-intensity focused ultrasound (HIFU): effective and safe therapy for hepatocellular carcinoma adjacent to major hepatic veins. *Eur Radiol* 2009; **19**: 437-445 [PMID: 18795303 DOI: 10.1007/s00330-008-1137-0]

10 **Jin C**, Zhu H, Wang Z, Wu F, Chen W, Li K, Su H, Zhou K, Gong W. High-intensity focused ultrasound combined with transarterial chemoembolization for unresectable hepatocellular carcinoma: long-term follow-up and clinical analysis. *Eur J Radiol* 2011; **80**: 662-669 [PMID: 20864286 DOI: 10.1016/j.ejrad.2010.08.042]

11 **Li C**, Zhang W, Zhang R, Zhang L, Wu P, Zhang F. Therapeutic effects and prognostic factors in high-intensity focused ultrasound combined with chemoembolisation for larger hepatocellular carcinoma. *Eur J Cancer* 2010; **46**: 2513-2521 [PMID: 20663659 DOI: 10.1016/j.ejca.2010.06.015]

12 **Cheung TT**, Chu FS, Jenkins CR, Tsang DS, Chok KS, Chan AC, Yau TC, Chan SC, Poon RT, Lo CM, Fan ST. Tolerance of high-intensity focused ultrasound ablation in patients with hepatocellular carcinoma. *World J Surg* 2012; **36**: 2420-2427 [PMID: 22699746 DOI: 10.1007/s00268-012-1660-7]

13 **Vogl TJ**, Naguib NN, Nour-Eldin NE, Rao P, Emami AH, Zangos S, Nabil M, Abdelkader A. Review on transarterial chemoembolization in hepatocellular carcinoma: palliative, combined, neoadjuvant, bridging, and symptomatic indications. *Eur J Radiol* 2009; **72**: 505-516 [PMID: 18835117 DOI: 10.1016/j.ejrad.2008.08.007]
14 **Golfieri R**, Cappelli A, Cucchetti A, Piscaglia F, Carpenzano M, Peri E, Ravaioli M, D'Errico-Grigioni A, Pinna AD, Bolondi L. Efficacy of selective transarterial chemoembolization in inducing tumor necrosis in small (& lt; 5 cm) hepatocellular carcinomas. *Hepatology* 2011; **53**: 1580-1589 [PMID: 21351114 DOI: 10.1002/hep.24246]
15 **Lu DS**, Yu NC, Raman SS, Lassman C, Tong MJ, Britten C, Durazo F, Saab S, Han S, Finn R, Hiatt JR, Busuttil RW. Percutaneous radiofrequency ablation of hepatocellular carcinoma as a bridge to liver transplantation. *Hepatology* 2005; **41**: 1130-1137 [PMID: 15841454 DOI: 10.1002/hep.20688]
16 **Mazzaferro V**, Battiston C, Perrone S, Pulvirenti A, Regalia E, Romito R, Sarli D, Schiavo M, Garbagnati F, Marchianò A, Spreafico C, Camerini T, Mariani L, Miceli R, Andreola S. Radiofrequency ablation of small hepatocellular carcinoma in cirrhotic patients awaiting liver transplantation: a prospective study. *Ann Surg* 2004; **240**: 900-909 [PMID: 15492574 DOI: 10.1097/01.sla.0000143301.56154.95]
17 **Schroeder T**, Sotiropoulos GC, Molmenti EP, Kuehl H, Cicinnati VR, Schmitz KJ, Kóbori L, Paul A, Mathé Z. Changes in staging for hepatocellular carcinoma after radiofrequency ablation prior to liver transplantation as found in the explanted liver. *Hepatogastroenterology* 2011; **58**: 2029-2031 [PMID: 22234073]
18 **Lau WY**, Lai EC, Leung TW. Current role of selective internal irradiation with yttrium-90 microspheres in the management of hepatocellular carcinoma: a systematic review. *Int J Radiat Oncol Biol Phys* 2011; **81**: 460-467 [PMID: 20888138 DOI: 10.1016/j.ijrobp.2010.06.010]
19 **Parikh S**, Hyman D. Hepatocellular cancer: a guide for the internist. *Am J Med* 2007; **120**: 194-202 [PMID: 17349437 DOI: 10.1016/j.amjmed.2006.11.020]

P- Reviewers: Lin ZY, Maruyama H **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.v19.i43.7500

World J Gastroenterol 2013 November 21; 19(43): 7500-7514
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (1): Hepatocellular carcinoma

Role of innate immunity in the development of hepatocellular carcinoma

Rajagopal N Aravalli

Rajagopal N Aravalli, Department of Radiology, University of Minnesota Medical School, Minneapolis, MN 55455, United States

Author contributions: Aravalli RN solely contributed to this paper.

Correspondence to: Rajagopal N Aravalli, PhD, Department of Radiology, University of Minnesota Medical School, MMC 292, Mayo Memorial Building, 420 Delaware Street S.E., Minneapolis, MN 55455, United States. arava001@umn.edu

Telephone: +1-612-6268174 Fax: +1-612-6265580

Received: August 27, 2013 Revised: September 29, 2013

Accepted: October 17, 2013

Published online: November 21, 2013

Abstract

Hepatocellular carcinoma (HCC) is the most common form of liver cancer worldwide. It is caused by a variety of risk factors, most common ones being infection with hepatitis viruses, alcohol, and obesity. HCC often develops in the background of underlying cirrhosis, and even though a number of interventional treatment methods are currently in use, recurrence is fairly common among patients who have had a resection. Therefore, whole liver transplantation remains the most practical treatment option for HCC. Due to the growing incidence of HCC, intense research efforts are being made to understand cellular and molecular mechanisms of the disease so that novel therapeutic strategies can be developed to combat liver cancer. In recent years, it has become clear that innate immunity plays a critical role in the development of a number of liver diseases, including HCC. In particular, the activation of Toll-like receptor signaling results in the generation of immune responses that often results in the production of pro-inflammatory cytokines and chemokines, and could cause acute inflammation in the liver. In this review, the current knowledge on the role of innate immune responses in the development and progression of HCC is examined, and emerging therapeutic strategies based on molecular mechanisms of HCC are discussed.

Key words: Hepatocellular carcinoma; Innate immunity; Toll-like receptor; Liver cancer; Inflammation

Core tip: Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide. Growing incidence of HCC has generated immense interest to understand the mechanisms of disease at the physiological, cellular and molecular levels with the hope of developing novel therapeutics for the treatment of HCC. In the past few years, it has become clear that innate immunity plays a critical role in the development and progression of HCC. In this review, these new developments and possibilities of developing novel therapeutic options based on this newly gained knowledge are discussed.

Aravalli RN. Role of innate immunity in the development of hepatocellular carcinoma. *World J Gastroenterol* 2013; 19(43): 7500-7514 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7500.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7500

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common form of liver cancer, and is the third leading cause of cancer-related deaths worldwide. It accounts for approximately 70%-80% of all primary liver cancer cases[1]. A variety of risk factors such as hepatitis viruses, vinyl chloride, tobacco, foodstuffs contaminated with aflatoxin B1 toxin, heavy alcohol intake, nonalcoholic fatty liver disease (NAFLD), diabetes, obesity, oral contraceptives, and hemochromatosis cause HCC[2]. Recurrence is quite common in patients who have had a resection, and survival rate is 30%-40% at

five years post-surgery[2]. As a recent surveillance, epidemiology, and end results study using the Medicare dataset of elderly patients in the United States has shown, in addition to the human loss, there is a substantial burden of health care expense of illness associated with HCC[3]. To underscore this point, the Centers for Disease Control has recently recommended one-time health screening for the entire generation born between 1945 and 1965. The dramatic rise in the incidence of HCC in Western countries in recent years has generated intense efforts to understand the mechanisms of disease at the physiological, cellular and molecular levels with the hope of developing novel therapeutics for the treatment of HCC.

PATHOPHYSIOLOGY OF HCC

The normal liver lobule is formed by hepatocytes, cholangiocytes and various non-parenchymal cells [Kupffer cells (KCs), liver sinusoidal endothelial cells (LSECs), and hepatic stellate cells (HSCs)]. Intrahepatic lymphocytes and liver-specific natural killer (NK) cells are also present in the sinusoidal lumen and perisinusoidal space of Disse[4]. Exposure to toxic substances and induction of immune responses in the liver can result in inflammation through the activation of KCs and HSCs, and can cause necrosis. In this process, liver fibrosis and cirrhosis may also occur. Even though the molecular basis for cancer-promoting effect of cirrhosis is unknown, the process of recurrent liver cell necrosis and regeneration with increased cell turn-over renders liver cells more sensitive to the adverse effects of other mutagenic agents[4]. Cirrhosis is responsible for significant morbidity and mortality, and is one of the most important risk factors for the development of HCC.

Carcinogenesis is a process that involves the transition of a normal cell into a preneoplastic lesion that develops into malignant tumor[4]. Growing evidence suggests that gradual accumulation of mutations and genetic changes in preoneoplastic hepatocytes causes malignant transformation that leads to the development of HCC[5,6]. Tissue environment also plays a critical role in tumor formation[7]. Interaction of different cell types in the tumor stroma with components of the extracellular matrix (ECM), either directly or indirectly result in the acquisition of an abnormal phenotype that causes this transformation. Tumor stroma consists of fibroblasts [also referred to as "cancer-associated fibroblasts (CAFs)"], macrophages (liver resident KCs and other tumor-infiltrating cells), leukocytes, HSCs, endothelial cells, pericytes, neutrophils, and dendritic cells (DCs)[8]. Each of these cells produces growth factors, cytokines, chemokines, free radicals, and other tumorigenic substrates that contribute to tumor initiation and progression[9].

In HCC, CAFs are involved in tumor initiation and progression. They produce epidermal growth factor (EGF), hepatocyte growth factor, fibroblast growth factor, interleukin 6 (IL-6), chemokine (C-X-C motif) ligand 12 (CXCL12), and matrix metalloproteases (MMP-3 and MMP-9)[10]. They also produce IL-8, cyclooxygenase 2, and secreted protein acidic rich in cysteine to recruit and stimulate macrophage production, which can further increase the activation of CAFs through the secretion of tumor necrosis factor-α (TNF-α) and platelet-derived growth factor (PDGF)[11,12]. Tumor-associated macrophages (TAMs) are "polarized" into M2 mononuclear phagocyte-like cells by various cytokines [IL-4, IL-10, and transforming growth factor β (TGF-β)] present in the tumor microenvironment[13]. These M2-like TAMs, in turn, express cytokines (IL-10 and TGF-β), chemokines (CCL17, CCL22 and CCL24), vascular endothelial growth factor (VEGF), and EGF to recruit regulatory T cells (Tregs) and to promote angiogenesis[14,15]. KCs are able to impair cluster of differentiation 8^+ ($CD8^+$) cytotoxic T lymphocyte (CTL)-mediated immune responses through programmed death ligand 1[16]. Moreover, when stimulated with pro-inflammatory cytokines (IL-1β, TNF-α and PDGF), KCs and HSCs produce osteopontin that plays a pivotal role in various cell signaling pathways, which promote inflammation, tumor progression and metastasis[17,18]. DCs process antigens and present them to infiltrating CTLs by expressing them on their cell surface. These antigen presenting cells (APCs) possess high endocytic activity, and therefore, are critical for the induction of immune surveillance in tumors, and for immune evasion[19]. Such tumor-antigen specific $CD8^+$ T cell responses were recently shown to suppress the recurrence of HCC[20].

In response to liver injury, HSCs transdifferentiate into myofibroblast-like cells and produce cytokines, chemokines, growth factors, and ECM[17]. This phenotypic transformation of HSCs is a key event in the development of hepatic fibrosis[21]. Hepatitis B virus (HBV)-encoded X protein, hepatitis C virus (HCV)-encoded non-structural proteins, MMP-9, PDGF, TGF-β, Janus kinase (JNK), insulin-like growth factor binding protein 5, and cathepsins (B and D) are potent inducers of HSC activation and proliferation that enhance liver fibrosis and carcinogenesis[17]. Endothelial cells express a variety of angiogenic receptors including VEGFR, EGFR, EGF homology domains-2 (Tie-2), PDGFR, and C-X-C chemokine receptors. Tumor-associated endothelial cells express high levels of TGF-β in HCC, which act as a chemoattractant for cluster of differentiation 105 (CD105, also known as endoglin), a type I cellular glycoprotein that is a port of the TGF-β receptor complex, to promote tumor angiogenesis[22]. It has been shown that $CD105^+$ endothelial cells express increased angiogenesis activity with greater resistance to chemo-therapeutic agents and inhibitors of angiogenesis[23].

Infiltration of T cells into the tumor microenvironment is an important regulator of cancer progression. In HCC tissues, $CD4^+/CD25^+$ Tregs impair proliferation and activation of CTLs, degranulation, and production of granzymes (A and B), and perforin[24]. In a recent study, the infiltration of these Tregs into the tumors of HCC patients was found to correlate with an increase in

tumor size[25]. Other studies have shown that low $CD8^+$ T cell counts and high Treg numbers correlate with poor prognosis in HCC patients, especially after resection[26-28]. More recently, a 14-immune gene signature that drives the infiltration of lymphocytes into tumor has been identified[29]. This signature, which includes pro-inflammatory cytokines [TNF-α and interferon (IFN)-γ] and chemokines (CXCL10, CCL5 and CCL2), is a good predictor of patient survival at early tumor stages. In addition, dysfunctional regulation of immune response by excessive neutrophil activity was also reported as a poor prognostic indicator after resection of HCC[30].

INNATE IMMUNITY

To successfully detect and eliminate invading pathogens by discriminating self from non-self, the mammalian immune system has developed mechanisms that can be divided into two distinct components: the innate immunity and the adaptive immunity. In most multicellular organisms, the highly conserved innate immune system provides the first line of defense to limit infection by detecting pathogens using germline-encoded proteins[31]. The adaptive immunity, present only in vertebrates, detects non-self through the recognition of peptide antigens by receptors expressed on the surface of B and T cells[32]. The adaptive responses are much more diverse than the innate responses as each B and T lymphocyte clone expresses a distinct antigen receptor that arose by somatic gene rearrangement through a process of evolution[33]. Most often innate immune responses emanate from the host cell surface receptor with the recognition of conserved structural motifs termed pathogen-associated molecular patterns (PAMPs) on the surface of microorganisms. Toll-like receptors (TLRs), Nucleotide-binding and oligomerization domain-like receptors (NLRs) and RIG-I-like receptors (RLRs) are the key receptors that recognize a variety of PAMPs[34]. While TLRs can recognize bacteria, viruses, fungi and protozoa, NLRs and RLRs detect bacteria and viruses, respectively. All these pattern recognition receptors (PRRs) generate innate immune responses, either by acting alone or in combination with other receptors. The focus of the review is on the role of TLRs as most of the current knowledge on the role of innate immunity in liver diseases has been obtained from studies on the TLRs.

TLR SIGNALING IN MAMMALIAN CELLS

Toll was first identified as a gene important in the establishment of dorsal-ventral orientation during embryonic development in the fruit fly *Drosophila melanogaster*[35]. Later, it was found that the Toll protein plays a critical role in the fly's immunity to fungal infections[35,36]. The first mammalian homolog of Toll, Toll-like receptor 4 (TLR4), was identified as a PRR required for adaptive immunity[37]. Subsequently, TLR4 and other TLRs were shown to play critical roles in generating innate immune responses against microbial pathogens in mammalian systems. To date, 11 human TLRs and 13 mouse TLRs have been identified, in addition to a number of TLRs in other vertebrates, that recognize a variety of PAMPs and trigger both innate and adaptive immune responses[32,38]. TLRs are membrane-bound proteins that contain varying numbers of extracellular leucine-rich repeats and a Toll-IL receptor (TIR) domain in the cytoplasmic region that are highly conserved (Figure 1). They recognize ligands through LRRs and transmit signals through their TIR domain *via* protein-protein interactions with cellular adaptor proteins triggering a cascade of signaling events such as phosphorylation of interleukin-1 receptor-associated kinase 1 (IRAK-1) and activation of Nuclear factor kappa B (NF-κB) or interferon regulatory factor 3 (IRF3), resulting in the production of immune mediators and IFN-inducible genes[39]. Thus, a direct or indirect association of a ligand with its cognate TLR serves as a signal to trigger an innate immune response. Each step along the TLR signaling pathways is tightly regulated by a complex mix of phosphorylation and targeted degradation, and sequestering of various signaling molecules is dependent upon the nature of the invading pathogen[40].

In general, vertebrate TLRs were classified into six distinct families based upon amino acid sequence homologies of LRRs[41]. Most mammalian cells express low levels of TLRs constitutively in a cell-type specific manner, and interestingly, they can be present in both membrane-bound and soluble forms. For example, the rainbow trout TLR5 is expressed constitutively as a membrane protein but upon induction with the bacterial flagellin, a soluble TLR5 is rapidly induced[42]. Normally, TLRs function as homodimers. However, some TLRs form heterodimers with other TLRs to recognize PAMPs. For instance, TLR2 associates either with TLR1 or TLR6 as a heterodimer to recognize triacylated lipoproteins and diacylated lipoproteins, respectively[32,38,40]. In addition, cellular membrane protein CD14 enhances the ligand recognition ability of TLR2[43].

TLR EXPRESSION IN THE LIVER

In the healthy liver, TLR expression is detectable only at very low levels[44]. Eight TLRs are expressed in the mammalian liver with varying levels of expression on hepatocytes, KCs, HSCs and LSECs[45]. These TLRs not only recognize microbial PAMPs but also the damage-associated molecular patterns (DAMPs) of dying host cells[46]. Even though hepatocytes express all TLRs, they are capable of responding to TLR2 and TLR4 ligands only, and these responses are very weak *in vivo*[47]. Under inflammatory conditions, however, hepatocyte response to TLR2 ligands was significantly enhanced but the response to TLR4 ligands was still not detectable in these cells[48]. LSECs express mRNAs of TLRs 1-9, and respond to various TLR ligands by expressing TNF-α, IL-6, and IFN-β[49,50]. KCs express all TLRs and respond to a variety of ligands by producing TNF-α

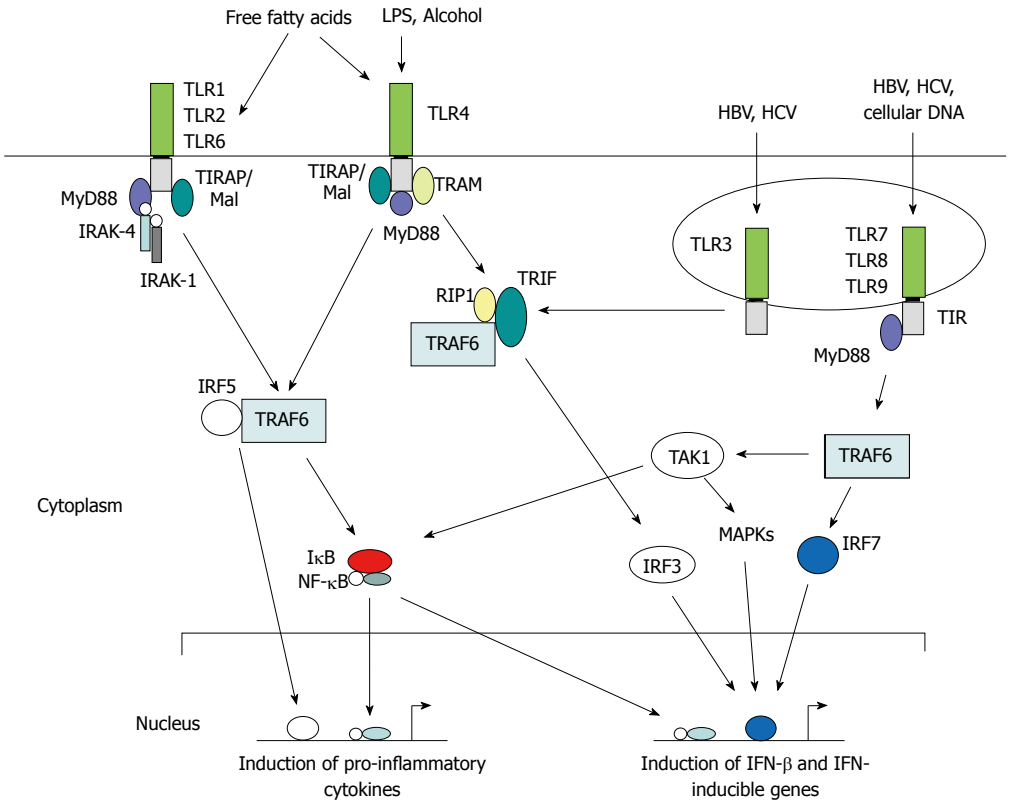


Figure 1 Toll-like receptor signaling in liver cells. Activation of a given TLR pathway is dependent on the nature of the stimulus. LPS: Lipopolysaccharide; TLR: Toll-like receptor; HBV: Hepatitis B virus; HCV: Hepatitis C virus; MyD88: Myeloid differentiation factor 88; IRAK: Interleukin-1 receptor-associated kinase; TIRAP: Toll-interleukin-1 receptor domain containing adaptor protein; TIR: Toll-interleukin-1 receptor; TRAM: TIR-domain-containing adapter molecule; IRF: Interferon regulatory factor; TRAF: Tumor necrosis factor receptor-associated factor; TAM: Tumor-associated macrophage; TAK: Transforming growth factor β-activated protein kinase; NF-κB: Nuclear factor kappa B; MAPK: Mitogen-activated protein kinase; TRIF: TIR-domain-containing adapter-inducing interferon-β; RIP: Receptor-interacting protein; IκB: Inhibitor of NF-κB; IFN: Interferon.

and IL-6[51,52]. When stimulated with ligands for TLRs 1, 2, 4 and 6, they produce IFN-γ and promote the proliferation of T cells[49]. In response to TLR3 and TLR4 ligands, they produce IFN-β, and for ligands against TLR1 and TLR8, they display a high level of major histocompatibility Ⅱ expression. HSCs express low levels of TLR4 and TLR9, but activation of TLR4 has been shown to induce the expression of TLR2 as well[49]. In human HSCs, TLR4 activation results in the production of CCL2, CCL3 and CCL4[53], and their expression of TGF-β was implicated in the promotion of hepatic fibrosis[54]. Activation of TLR9 by DAMPs induces the differentiation of HSCs and increases the production of collagen[55].

Among the non-parenchymal cells, hepatic DCs can be classified into different subsets as plasmacytoid DCs, myeloid DCs, lymphoid DCs, natural killer DCs, and a mixture of lymphoid and myeloid DCs[56]. Differential expression of TLRs in these hepatic DCs varies according to the subset type and the species type. For example, in humans, pDCs express TLRs 1, 3 and 7 only, whereas other DC subsets express all TLRs except TLR9[57]. On the other hand, murine pDCs express TLRs 2, 4, 7 and 9 but not TLR3[58].

Hepatic lymphocyte population, which accounts for about 25% of non-parenchymal cells consists of B cells, NK cells, NKT cells, αβT cells and γδT cells[59]. In general, T cells are activated indirectly by TLRs through APCs[60], and NK cells that express TLRs 1 to 9, but not TLR5. They respond to various TLR ligands by producing IL-12[61]. Interestingly, expression of TLRs in B cells has no effect on antibody production, and on B cell memory responses[62]. However, TLR3 and TLR9 activation in $CD4^+$ T cells enhances their proliferation[63], and TLR2 serves as a co-stimulatory receptor for antigen-specific T cell development and participates in T cell memory[64].

INVOLVEMENT OF TLRS IN LIVER DISEASES

Significant amount of evidence in recent years has demonstrated the involvement of TLRs in the pathogenesis of various liver diseases. Most of this evidence comes from the overexpression of TLRs, activation of TLRs causing enhanced disease in animal models, single nucleotide polymorphisms (SNPs) in TLR-coding genes and their adapters linked to disease susceptibility, and TLR knockout mice being protected from disease[40]. Three most common risk factors for the development of HCC for which TLR involvement has demonstrated to play a critical role

in the disease pathogenesis are discussed in this section.

Viral hepatitis

Chronic hepatitis virus infection that affects almost half a billion people worldwide is a major risk factor for HCC. The infectivity of a given virus type varies according to the geographical location[2]. Co-infection with one or more viruses may also occur contributing to a higher risk of HCC, albeit it is rare[35]. HCV is the most common blood-borne infection in the United States, with nearly 20% of chronically infected individuals developing cirrhosis and HCC[65]. It is generally acknowledged that the humoral antibody response contributes to the clearance of circulating HBV particles and the prevention of viral spread within the host while the cellular immune response eliminates infected cells[66]. The T cell response to the HBV is vigorous, polyclonal and multispecific in acutely infected patients who successfully clear the virus, and relatively weak and narrowly focused in chronically infected patients, suggesting that clearance of HBV is T cell dependent[66]. Persistent HBV infection is characterized by chronic liver cell injury, regeneration, inflammation, widespread DNA damage and insertional deregulation of cellular growth control genes, which, collectively, lead to cirrhosis of the liver and HCC[66]. Other factors that could contribute to viral persistence are immunological tolerance, mutational epitope inactivation, T-cell receptor antagonism, incomplete down-regulation of viral replication, and infection of immunologically privileged tissues[66]. However, these pathways become apparent only in the setting of an ineffective immune response, which therefore, is the fundamental underlying cause[66]. In infected cells, the HBV capsid induces cytokine production *via* TLR2 activation. It was hypothesized that TLR2 activation is involved in viral clearance based on the observation that the administration of adefovir and entricitabine in HBV patients resulted in the up-regulation of TLR2 and reduction in the viral load[46]. In HepG2 cells, HBV triggers the production of cholesterol-metabolism genes *via* the TLR2 pathway[67], and inflammatory stress exacerbated hepatic cholesterol accumulation these cells and in mice by disrupting the PPAR-LXR-CYP7A1/ABCA1-mediated bile acid synthesis and cholesterol efflux[68]. Interestingly in HBV transgenic mice, activation of TLRs 3-5, 7, and 9, but not TLR2, inhibited HBV replication *via* IFN-α/β induction[69].

When immune responses were compared in macrophages of patients who spontaneously cleared HCV with those who were chronically infected, it was found that the TLR3 expression was significantly up-regulated in the former group[65]. Individuals who cleared the virus had an elevated expression of IFN-β and higher rate of STAT-1 phosphorylation. A significant association of intronic TLR3 SNP (rs13126816) in the clearance of HCV and the expression of TLR3 was found in this study, suggesting that an elevated innate immune response enhances HCV clearance and may offer a potential therapeutic option to increase viral clearance[65]. TLR2, in combination with TLR1 and TLR8, recognizes core and nonstructural 3 proteins of HCV in immune cells such as macrophages and monocytes, and triggers the production of pro-inflammatory immune mediators TNF-α, IL-6, IL-8, IL-10 and IL-1β[70]. On the other hand, HCV NS5 protein is recognized by TLR4 in both hepatocytes and B cells[71]. Prolonged activation of KCs in the HCV-infected liver by HCV-encoded proteins causes persistent inflammation resulting in severe liver damage and cancer[72]. It was reported recently that the expression of TLR2 and TLR4 was highly elevated in peripheral blood monocytes of HCV patients, and that the number of Tregs was significantly higher in these chronically infected individuals[73]. Similar correlation of TLR2 and TLR4 expression, and Treg numbers was also reported in HBV patients[74]. In a separate study, a strong correlation was also observed between TLR2/4 expression and TNF-α production causing hepatic inflammation in HCV patients[75]. Chronic infection with HBV and/or HCV, and imbalanced immunity contributes to severe inflammation, while TLR activation during this process might be a critical factor of this infection-induced prolonged inflammation. Activation of innate immune responses by viral proteins, and the interaction of HCV with cellular proteins to evade host's immune responses as well as the role of SNPs in TLRs, their adaptors and cytokine genes in altering these immune responses have been extensively reviewed in the literature[76-78].

Alcoholic liver disease

Excessive alcohol consumption that causes alcoholic liver disease (ALD) is a major risk factor for HCC worldwide[79]. ALD is an umbrella term used to describe a broad spectrum of liver abnormalities caused by alcohol that include simple steatosis, alcoholic hepatitis, fibrosis and cirrhosis, which can progress to HCC. The pathogenesis of ALD is characterized by processes such as ethanol metabolism-associated oxidative damage, glutathione depletion, abnormal methionine metabolism, ethanol-mediated induction of leakage of gut endotoxins, and inflammation[79]. Liver inflammation is known to occur with exposure to a variety of agents, including metabolites of alcohol[80]. If hepatic metabolism is impaired to any degree and fails to convert drugs and chemicals to non-reactive or non-immunogenic substances, the metabolic intermediates formed in hepatic tissues may cause liver damage[81]. In such cases, KCs and other cell types release cytokines and chemokines that result in the inflammation of the liver. This reaction coupled with deregulated hepatocyte proliferation can contribute to the pathogenesis of HCC[81].

Simple steatosis is a benign condition that progresses to alcoholic steatohepatitis (ASH) in about 10%-20% of cases and is associated with inflammation and liver injury caused by the innate immune responses[82]. Both MyD88-dependent and MyD88-independent pathways are activated in ASH, and studies with animal models have demonstrated the up-regulation of inflammatory cytokines

in the serum, and activation of IFN and IFN-responsive genes in ASH[83,84]. Blocking of IL-1 receptor, which acts through MyD88, in advanced ASH was shown to confer protective effect in mice suggesting that IL-1 inhibitors may be used in the treatment of ALD[85].

Excessive alcohol consumption increases gut permeability and the translocation of bacteria-derived lipopolysaccharide (LPS, also known as endotoxin) from the gut to the liver[86]. In KCs, LPS interacts with TLR4 causing oxidative stress, and the production of pro-inflammatory cytokines and reactive oxygen species (ROS) that induce hepatocellular damage[85,87]. However, this effect was not abrogated in MyD88-deficient mice suggesting that MyD88-independent pathways are involved in NF-κB activation by alcohol[83]. Even though IRF-3 activation was not affected by chronic alcohol treatment, IRF-7 and IRF-3-inducible genes expression was significantly induced in KCs of alcohol-fed wild type mice[83]. The activation of TLR4 has also been demonstrated to occur during the alcohol and HCV synergism[88]. Furthermore, alcohol activates complements C3 and C5, following the production of TNF-α, and induces hepatocyte injury[89,90]. In addition to Gram-negative bacteria that produce LPS, gut is home to a large number of other microorganisms belonging to eukaryotes, prokaryotes, archaea, and viruses[91]. It is therefore likely that Gram-positive bacteria which produce lipoproteins and activate TLR2 signaling, and viruses that activate TLRs 3, 7, 8 and 9 may also leak from the gut to the liver. However, little is known about the activation of these TLRs during alcoholic liver injury.

Nonalcoholic steatohepatitis

NAFLD is the most common chronic liver disease that affects both adults and children worldwide[92]. Like ALD, NAFLD includes a broad spectrum of liver abnormalities ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), which can progress to cirrhosis and HCC. Of these, NASH is characterized by hepatocyte injury, inflammation, and fibrosis[92-94]. Most of these disease conditions are diagnosed at a late stage, and some patients also present for the first time with cirrhosis[92]. The development of cirrhosis is significantly higher in individuals with NASH when compared to patients with simple steatosis[92-94]. Consequently, liver-related mortality is also significantly higher in NASH patients.

NASH shares immunological characteristics with ASH such as the activation of innate immune responses, crosstalk between steatosis and inflammation, activation of TLR4 by LPS and fatty acids, complement activation, production of proinflammatory immune mediators, and alteration in NK and NKT cell number and activity[95,96]. In addition, activation of KCs in response to gut microbiota, activation of TLR signaling *via* both MyD88-dependent and -independent pathways, up-regulation of type I IFN and IFN-responsive genes occur in ASH and NASH[97-100]. However, recent studies have shown that NASH differs from ASH in certain aspects: insulin resistance, crosstalk between adipose tissue and the liver, dependence on MyD88 signaling, differential effects in mice due to MyD88 and IL-1 deficiency, and activation of inflammasome[96].

Progression of NASH is associated with the recruitment of T cells and T1 response leading to inflammation[101], and hepatic expression levels of inflammatory mediators are modified in morbidly obese patients even without pathohistological manifestations[102]. Accumulating evidence strongly suggests that inflammation causes the progression of NAFLD to NASH, and that innate immunity is involved in the inflammatory response. While TLR2 and TLR9 pathways are critical for the development of NAFLD[103], deletion of either TLR4 or its co-receptor MD-2 dampened (but not abolished) necro-inflammatory activity of steatosis and fibrosis in a mouse model of NASH[104]. Furthermore, in NASH, gut microorganisms enter the liver because of the leakage in the intestinal mucosal barrier resulting in inflammation, due to the activation of TLR signaling by intestinal bacteria and their products such as LPS[45,93]. It is apparent from these reports that the activation of TLR signaling is an important factor in causing inflammation in NASH, and could be a crucial factor in the progression of NASH to cirrhosis. It was reported that the activation of TLR4 signaling and up-regulation of CD14 resulted in higher responsiveness to LPS and saturated fatty acids in KCs, and resulted in hepatic inflammation and liver complications in NASH patients[98,105].

Hepatocarcinogenesis

Chronic liver damage caused by excessive inflammation due the exposure to various risk factors often results in the development of fibrosis-associated HCC[106]. Since the stimulation of TLR signaling pathways result in the production of proinflammatory immune mediators, it is likely that TLRs are involved in development and progression of hepatocarcinogenesis as well. The activation of TLR signaling in liver diseases leads to the activation of NF-κB and JNK pathways that are critical mediators of tumor-associated cytokine production. NF-κB activation induces the proliferation of tumor cells through the production of TNF-α[107]. Tumorigenic effect of JNK in HCC is mediated through the regulation of molecules involved in cell proliferation such as MMPs and cyclins[108]. Studies in animal models of HCC have shown that ROS-mediated JNK activation is critical for tumor development[109], and that elevated ROS levels induce hepatocyte cell death[110]. Moreover, hepatocyte-specific knockdown of the NF-κB inhibitor IκB kinase subunit NF-κB essential modulator causes spontaneous development of HCC in mice[111]. However, interestingly, hepatocyte-specific TGF-β-activated protein kinase 1-deficient mice had spontaneous hepatocyte cell death, compensatory proliferation, inflammatory cell infiltration, and fibrosis in the liver[102,112]. Collectively, these results demonstrate the involvement of NF-κB-mediated downstream molecules in cellular homeostasis and cancer development in the liver.

TLR POLYMORPHISMS AND THE RISK OF DEVELOPING HCC

Dysregulation of TLR expression may shift the balance between the production of pro- and anti-inflammatory cytokines, and will have a profound effect on the risk of infection, chronic inflammation and cancer. Recently, Nishalke *et al*[113] showed that the frequency of TLR2 -196 to -714del allele was significantly higher in HCV-associated HCC patients than in HCV-infected individuals demonstrating that this deletion plays a role in HCC development. In addition, a number of SNPs have also been identified in every TLR gene. Some of these have been shown to enhance the susceptibility of various cancers in humans[114]. However, only a few reports have been published on the role of TLR SNPs in the pathogenesis of HCC. In one such study, Zhang *et al*[115], conducted a genome-wide genotyping of 440794 SNPs in chronic HBV carriers: 355 with HCC and 360 without HCC. They found that one intronic SNP rs17401966 present in the *KIF1B* gene on chromosome 1p36.22 was highly associated with HBV-associated HCC[115]. In a recent study, Junjie *et al*[116] investigated the association between SNPs of *TLR2* and *TLR9* genes, and the susceptibility of HCC in a cohort of 211 patients that included 172 HBV carriers. They found that two TLR2 SNPs rs3804099 C/T and rs3804100 C/T present in the same exon of TLR2 were associated with HCC, whereas TLR9 SNPs have no role in tumor development[116]. Additional studies are needed to fully understand the involvement of various TLR SNPs in hepatocarcinogenesis.

MODULATION OF TLR SIGNALING TO ALLEVIATE LIVER INFLAMMATION AND CANCER

Overwhelming evidence on the involvement of TLR signaling pathways in many human diseases has led to the efforts to develop TLRs as vaccine adjuvants[117,118] and as therapeutic targets[119-121]. As discussed earlier, chronic inflammation, which plays a critical role in the progression of liver diseases and in the development of human cancers, is mediated by TLR activation. Hence, modulation of TLR pathways using various drugs, antibodies, microRNAs (miRNAs), and small molecules that function as TLR agonists and antagonists to reduce liver inflammation and to prevent the progression of liver diseases towards cancer is a promising strategy to combat HCC.

TLR agonists and antagonists

Initial efforts on the modulation TLR signaling with small TLR mimetics that act as agonists have produced mixed results. Using the TLR2 agonist Pam2Cys as a stimulant *in vitro*, it was shown that hepatoma cell lines HuH7 and HepG2 respond to HBV infection by producing IL-8 and by inhibiting viral replication[122]. However, in *in vivo* studies with HBV transgenic mice, it was found that ligands specific for TLRs 3, 4, 5, 7 and 9, but not TLR2, inhibited HBV replication in the liver[69]. On the other hand, intravenous administration of the TLR7 agonist isatoribine significantly reduced viral RNA in HCV patients independent of viral genotype[123]. The decrease in viral load in this study coincided with markers indicating a heightened antiviral immune state. In similar experiments, Weeratna *et al*[118] and Ma *et al*[124] tested TLR7 agonist R-848 and CpG oligodeoxynucleotides containing CpG motifs (CpG ODN) that act as the TLR9 agonists as adjuvants for HBV vaccination in mice using the HBV surface antigen (HBsAg) as a model antigen. This study was based on the notion that the immunostimulatory sequences (ISS) containing CpG motifs would elicit strong Th1 immune responses[125]. In their studies, both these groups found that the TLR agonists trigger immune responses that are beneficial to the host[118,124]. Along similar lines, Dynavax Corporation has developed a commercial vaccine (Heplisav) against HBV by combining a synthetic ISS sequence called ISS-1018 that acts as TLR9 agonist with the recombinant HBsAg. This vaccine induced seroprotective responses when administered in HBV patients[126]. Another CpG ODN containing TLR9, CPG7909, was also successfully tested as an adjuvant to HBV vaccine and is currently undergoing a phase 2 clinical trial[125,127].

In contrast to these TLR agonists, only a few studies have reported successful inhibition of TLR signaling using antagonists. For example, lipid A mimetics that bind directly to TLR4-MD2 were shown to inhibit LPS-mediated activation of TLR4 signaling both *in vitro* and *in vivo*[128]. TAK-242, another mimetic that targets the intracellular domain of TLR4, inhibited TLR4-mediated production of nitric oxide and TNF-α in murine and human cells by targeting TLR4-CD4 chimeric receptors[129]. Interestingly, however, TAK-242 had no effect on NF-κB activation mediated by MyD88, TIRAP, TRIF or TRAM. Recently, Cowden *et al*[130] tested two inhibitors of histamine H4 receptor that interacts with TLR4 and found that they reduced TNF-α production and LPS-induced inflammation in mouse livers.

In addition to the strategy of inhibiting TLRs, efforts are also being made to selectively inhibit their adaptors and downstream signaling molecules[131]. In such studies, Compound 4a inhibited the interaction of MyD88 protein with the TIR domain of TLRs and type I IL-1 receptor[132], whereas MyD88 inhibitors ST2825 and RO0884 blocked the recruitment of IRAK-1 and IRAK-4 in human cells[133,134]. Additionally, ST2825 also suppressed B cell proliferation and differentiation[133]. However, the efficacy of these molecules in reducing inflammation in the liver is yet to be determined.

miRNAs

MiRNAs are a class of small, noncoding RNAs that act as key regulators of many cellular processes. During the past decade, it has been well established that the aberrant expression of a large number of miRNAs correlate with

disease severity and poor prognosis of HCC[135-144]. The expression of these "miRNA signatures" are unique for a given disease stage, and were useful in identifying patients with HCC who are likely to develop metastases and recurrence. In recent years, efforts to identify miRNAs involved in the regulation of innate immune genes has resulted in the identification of a few promising miRNA candidates that can be exploited for therapeutic purposes. For example, overexpression of miR-155 which is directly involved in the regulation of more than 30 innate immune genes[145] results in the significant reduction of MyD88 expression and IL-8 production induced by *Helicobacter pylori*[146]. Interestingly, in bone marrow-derived macrophages and RAW264.7 cells, IL-10 inhibits miR-155 expression after LPS stimulation and dampens inflammatory immune responses in a STAT-3 dependent manner[147].

When THP-1 monocytes were treated with ligands for TLRs 2, 4 and 5, production of TNF-α correlated inversely with the up-regulation of miR-146a[148], and when the tragets of miR-146a IRAK-1 or TRAF6 were knocked down, inflammatory responses to TLR2, TLR4 and TLR5 ligands were significantly reduced in these cells suggesting a role for miR-146a in LPS-induced cross-tolerance. Similarly, miR-132 and miR-212 were shown to be involved in TLR2-mediated cross-tolerance through IRAK4 modulation[149]. In addition, miR-146a disrupts the translation of TLR4-induced TNF-α production in these cells[150]. miR-146b exerts this effect in monocytes by negatively regulating TLR4 *via* a IL-10-mediated STAT3 dependent loop[151]. Another study on the expression profiling of endotoxin responsive genes in human monocytes has revealed that miR-146 regulates TLR and cytokine signaling in a NF-κB-dependent manner through a negative feedback regulation loop involving the down-regulation of IRAK-1 and TRAF6 protein levels[152]. In THP-1 cells, miR-146a also regulates TLR2 signaling by reducing IRAK-1 and phosphorylated IκBα expression[153].

In another study, miR-181a expression was found to be significantly elevated in mice stimulated with LPS and streptozotocin, which correlated strongly with the expression of inflammatory factors TNF-α, IL-6, IL1β in macrophages suggesting that the inhibition of miR-181a could reduce TLR4-induced inflammation[154]. After stimulation with LPS, miR-92a significantly inhibited the activation of JNK/c-Jun pathway and the production of inflammatory cytokines in macrophages by directly targeting mitogen-activated protein kinase kinase 4[155], demonstrating that miR-92a is a negative regulator of TLR-triggered immune responses.

In other studies, Benakanakere *et al*[156] demonstrated that miR-105 binds directly to TLR2 and regulates its expression. When keratinocytes were challenged by a TLR2 agonist or when miR-105 was overexpressed, a strong inverse correlation between miR-105 expression and TLR2 protein levels was observed. Similar to miR-105, let-7i was recently shown to bind directly to TLR4 in cholangiocytes and to control its expression by translational repression[157]. In this study, let-7i mimics inhibited *Cryptospiridium parvum* induced TLR4 production. In dendritic cells of mice with colitis, the expression of miR-10a was found to be negatively regulated by the intestinal microbiota, which correlated inversely with the production of IL-12/IL-23p40[158]. This finding suggests that miR-10a targets IL-12/IL-23p40 to maintain immune homeostasis.

When stimulated with ligands for TLRs 2-4 miR-147 is induced in murine macrophages[159]. Its expression was found to be greater after the activation of TLR4 than that of TLR2 or TLR4, and was dependent on both MyD88 and TRIF. *In vivo*, TLR stimulation induced the expression of miR-147 as a negative-feedback loop mechanism to prevent excessive inflammatory responses[159]. Likewise, miR-145 targets the adaptor protein MAL, which facilitates the interaction between TLR and TLR4 with MyD88, and inhibits its expression[152]. Recently, Wendlandt *et al*[160] demonstrated that miR-200b and miR-200c reduced the expression of MyD88 but had no effect TLR4, IRAK-1 and TRAF6 in HEK293 cells. When miR-200b and miR-200c mimics were overexpressed, LPS-induced expression of IL-6, TNF-α, and CXCL9 was diminished, suggesting that these miRNAs regulate TLR4 signaling *via* MyD88-dependent manner[160]. Up-regulated miR-19a and miR-19b were able to inhibit the expression of suppressor of cytokine signaling 1, a gene important in the negative regulation of TLR signaling[161].

Programmed cell death 4 (PDCD4) is a pro-inflammatory protein that suppresses IL-10 production and activates NF-κB. Sheedy *et al*[162] found that PDCD4-deficient mice are protected from LPS-induced death and demonstrated that miR-21 targets PDCD4 after LPS stimulation to negatively regulate TLR4 pathway. Recently, Fabbri *et al*[163] reported that miR-21 and miR-29b secreted by lung cancer cells in exosomes function as TLR ligands. These miRNAs bind to and activate TLR7 and TLR8 signaling pathways in peritoneal macrophages cells, causing NF-κB activation and secretion of pro-metastatic inflammatory cytokines[163]. Secreted miR-21 was also found in the serum of HCC patients and in individuals with chronic hepatitis indicating that miR-21 is a key factor in hepatocarcinogenesis[164]. Several other circulating miRNAs have also been identified in the serum of patients with HCC[165-167]. Further studies are needed to determine whether any of these miRNAs can be used to modulate the TLR signaling pathways, and to alleviate inflammation in liver tissues.

CONCLUSION

Hepatocarcinogenesis is a multifactorial disease in which the expression of a large number of genes, proteins, and other molecules from diverse cellular processes is altered[2]. Growing evidence suggests that inflammation plays a critical role in the progression of liver diseases to HCC. Therefore, the use of combination therapy that

targets multiple different steps and pathways, rather than a single test or a set of tests, to reduce inflammation is an appropriate therapeutic strategy to combat the development of HCC. Positive therapeutic outcomes achieved with sorafenib that can inhibit receptor tyrosine kinases of multiple signaling cascades[168], as well as evidence that a single molecule miR-26a can significantly reduce HCC without any toxicity[169] demonstrate the success of this multi-pronged approach.

Despite recent advances in the use of miRNAs either as antagomirs or in replacement therapy, the field of miRNA therapeutics for HCC is still in its infancy. Only a handful of successful outcomes have been reported so far[135]. One such success story was that of MiraVasen, which inhibited the activity of miR-122 and suppressed HCV viraemia with no evidence for viral resistance or side effects in chimpanzees[170]. This was the first miRNA-based therapeutic drug developed to treat a liver disease, and is currently being tested by Santaris Pharma (Hørsholm, Denmark) in phase 2 clinical trials[171]. The first successful demonstration of miRNA replacement to restore the expression levels of a down-regulated miRNA by delivery to the tumor was reported using a miR-26a-encoding AAV vector in a mouse model of HCC[169]. More recently, another miRNA down-regulated in HCC, miR-34a, has been successfully tested using this approach an orthotopic model of HCC[172]. In latter two cases, significant tumor reduction, dramatic protection from disease progression without toxicity, and prolonged survival of animals has been reported. While these reports were aimed to restore a loss of function, similar success has not yet been achieved for HCC treatment using the approaches to inhibit miRNA expression levels. In future investigations, it is important to exercise caution while designing clinical trials with miRNA mimics and TLR inhibitors for targeting multiple genes relevant to the liver diseases because of the concerns about potential toxicity in normal tissues, and as they can cause severe detrimental effects due to imbalances in TLR expression, and cellular functions that could cause immune paralysis leading to development of HCC.

REFERENCES

1 **Nordenstedt H**, White DL, El-Serag HB. The changing pattern of epidemiology in hepatocellular carcinoma. *Dig Liver Dis* 2010; **42** Suppl 3: S206-S214 [PMID: 20547305 DOI: 10.1016/S1590-8658(10)60507-5]

2 **Aravalli RN**, Steer CJ, Cressman EN. Molecular mechanisms of hepatocellular carcinoma. *Hepatology* 2008; **48**: 2047-2063 [PMID: 19003900 DOI: 10.1002/hep.22580]

3 **Lang K**, Danchenko N, Gondek K, Shah S, Thompson D. The burden of illness associated with hepatocellular carcinoma in the United States. *J Hepatol* 2009; **50**: 89-99 [PMID: 18977551 DOI: 10.1016/j.jhep.2008.07.029]

4 **Severi T**, van Malenstein H, Verslype C, van Pelt JF. Tumor initiation and progression in hepatocellular carcinoma: risk factors, classification, and therapeutic targets. *Acta Pharmacol Sin* 2010; **31**: 1409-1420 [PMID: 20953207 DOI: 10.1038/aps.2010.142]

5 **El-Serag HB**, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. *Gastroenterology* 2007; **132**: 2557-2576 [PMID: 17570226 DOI: 10.1053/j.gastro.2007.04.061]

6 **Farazi PA**, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. *Nat Rev Cancer* 2006; **6**: 674-687 [PMID: 16929323 DOI: 10.1038/nrc1934]

7 **Yang JD**, Nakamura I, Roberts LR. The tumor microenvironment in hepatocellular carcinoma: current status and therapeutic targets. *Semin Cancer Biol* 2011; **21**: 35-43 [PMID: 20946957 DOI: 10.1016/j.semcancer.2010.10.007]

8 **Schrader J**, Iredale JP. The inflammatory microenvironment of HCC - the plot becomes complex. *J Hepatol* 2011; **54**: 853-855 [PMID: 21185341 DOI: 10.1016/j.jhep.2010.12.014]

9 **Wu SD**, Ma YS, Fang Y, Liu LL, Fu D, Shen XZ. Role of the microenvironment in hepatocellular carcinoma development and progression. *Cancer Treat Rev* 2012; **38**: 218-225 [PMID: 21763074 DOI: 10.1016/j.ctrv.2011.06.010]

10 **Pietras K**, Ostman A. Hallmarks of cancer: interactions with the tumor stroma. *Exp Cell Res* 2010; **316**: 1324-1331 [PMID: 20211171 DOI: 10.1016/j.yexcr.2010.02.045]

11 **Mueller L**, Goumas FA, Affeldt M, Sandtner S, Gehling UM, Brilloff S, Walter J, Karnatz N, Lamszus K, Rogiers X, Broering DC. Stromal fibroblasts in colorectal liver metastases originate from resident fibroblasts and generate an inflammatory microenvironment. *Am J Pathol* 2007; **171**: 1608-1618 [PMID: 17916596 DOI: 10.2353/ajpath.2007.060661]

12 **Zhang Y**, Yang B, Du Z, Bai T, Gao YT, Wang YJ, Lou C, Wang FM, Bai Y. Aberrant methylation of SPARC in human hepatocellular carcinoma and its clinical implication. *World J Gastroenterol* 2012; **18**: 2043-2052 [PMID: 22563191 DOI: 10.3748/wjg.v18.i17.2043]

13 **Mantovani A**, Sica A, Allavena P, Garlanda C, Locati M. Tumor-associated macrophages and the related myeloid-derived suppressor cells as a paradigm of the diversity of macrophage activation. *Hum Immunol* 2009; **70**: 325-330 [PMID: 19236898 DOI: 10.1016/j.humimm.2009.02.008]

14 **Mantovani A**, Germano G, Marchesi F, Locatelli M, Biswas SK. Cancer-promoting tumor-associated macrophages: new vistas and open questions. *Eur J Immunol* 2011; **41**: 2522-2525 [PMID: 21952810 DOI: 10.1002/eji.201141894]

15 **Movahedi K**, Laoui D, Gysemans C, Baeten M, Stangé G, Van den Bossche J, Mack M, Pipeleers D, In't Veld P, De Baetselier P, Van Ginderachter JA. Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes. *Cancer Res* 2010; **70**: 5728-5739 [PMID: 20570887 DOI: 10.1158/0008-5472.CAN-09-4672]

16 **Wu K**, Kryczek I, Chen L, Zou W, Welling TH. Kupffer cell suppression of CD8+ T cells in human hepatocellular carcinoma is mediated by B7-H1/programmed death-1 interactions. *Cancer Res* 2009; **69**: 8067-8075 [PMID: 19826049 DOI: 10.1158/0008-5472.CAN-09-0901]

17 **Leonardi GC**, Candido S, Cervello M, Nicolosi D, Raiti F, Travali S, Spandidos DA, Libra M. The tumor microenvironment in hepatocellular carcinoma (review). *Int J Oncol* 2012; **40**: 1733-1747 [PMID: 22447316]

18 **Ramaiah SK**, Rittling S. Pathophysiological role of osteopontin in hepatic inflammation, toxicity, and cancer. *Toxicol Sci* 2008; **103**: 4-13 [PMID: 17890765 DOI: 10.1093/toxsci/kfm246]

19 **Lin A**, Schildknecht A, Nguyen LT, Ohashi PS. Dendritic cells integrate signals from the tumor microenvironment to modulate immunity and tumor growth. *Immunol Lett* 2010; **127**: 77-84 [PMID: 19778555 DOI: 10.1016/j.imlet.2009.09.003]

20 **Hiroishi K**, Eguchi J, Baba T, Shimazaki T, Ishii S, Hiraide A, Sakaki M, Doi H, Uozumi S, Omori R, Matsumura T, Yanagawa T, Ito T, Imawari M. Strong CD8(+) T-cell responses against tumor-associated antigens prolong the recurrence-free interval after tumor treatment in patients with hepatocellular carcinoma. *J Gastroenterol* 2010; **45**: 451-458 [PMID:

19936602 DOI: 10.1007/s00535-009-0155-2]
21 **Friedman SL**. Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008; **134**: 1655-1669 [PMID: 18471545 DOI: 10.1053/j.gastro.2008.03.003]
22 **Benetti A**, Berenzi A, Gambarotti M, Garrafa E, Gelati M, Dessy E, Portolani N, Piardi T, Giulini SM, Caruso A, Invernici G, Parati EA, Nicosia R, Alessandri G. Transforming growth factor-beta1 and CD105 promote the migration of hepatocellular carcinoma-derived endothelium. *Cancer Res* 2008; **68**: 8626-8634 [PMID: 18922939 DOI: 10.1158/0008-5472.CAN-08-1218]
23 **Xiong YQ**, Sun HC, Zhang W, Zhu XD, Zhuang PY, Zhang JB, Wang L, Wu WZ, Qin LX, Tang ZY. Human hepatocellular carcinoma tumor-derived endothelial cells manifest increased angiogenesis capability and drug resistance compared with normal endothelial cells. *Clin Cancer Res* 2009; **15**: 4838-4846 [PMID: 19638466]
24 **Fu J**, Xu D, Liu Z, Shi M, Zhao P, Fu B, Zhang Z, Yang H, Zhang H, Zhou C, Yao J, Jin L, Wang H, Yang Y, Fu YX, Wang FS. Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. *Gastroenterology* 2007; **132**: 2328-2339 [PMID: 17570208 DOI: 10.1053/j.gastro.2007.03.102]
25 **Lee WC**, Wu TJ, Chou HS, Yu MC, Hsu PY, Hsu HY, Wang CC. The impact of CD4+ CD25+ T cells in the tumor microenvironment of hepatocellular carcinoma. *Surgery* 2012; **151**: 213-222 [PMID: 21975289 DOI: 10.1016/j.surg.2011.07.029]
26 **Wang F**, Jing X, Li G, Wang T, Yang B, Zhu Z, Gao Y, Zhang Q, Yang Y, Wang Y, Wang P, Du Z. Foxp3+ regulatory T cells are associated with the natural history of chronic hepatitis B and poor prognosis of hepatocellular carcinoma. *Liver Int* 2012; **32**: 644-655 [PMID: 22118340 DOI: 10.1111/j.1478-3231.2011.02675.x]
27 **Chen KJ**, Zhou L, Xie HY, Ahmed TE, Feng XW, Zheng SS. Intratumoral regulatory T cells alone or in combination with cytotoxic T cells predict prognosis of hepatocellular carcinoma after resection. *Med Oncol* 2012; **29**: 1817-1826 [PMID: 21678026 DOI: 10.1007/s12032-011-0006-x]
28 **Mathai AM**, Kapadia MJ, Alexander J, Kernochan LE, Swanson PE, Yeh MM. Role of Foxp3-positive tumor-infiltrating lymphocytes in the histologic features and clinical outcomes of hepatocellular carcinoma. *Am J Surg Pathol* 2012; **36**: 980-986 [PMID: 22446942 DOI: 10.1097/PAS.0b013e31824e9b7c]
29 **Chew V**, Chen J, Lee D, Loh E, Lee J, Lim KH, Weber A, Slankamenac K, Poon RT, Yang H, Ooi LL, Toh HC, Heikenwalder M, Ng IO, Nardin A, Abastado JP. Chemokine-driven lymphocyte infiltration: an early intratumoural event determining long-term survival in resectable hepatocellular carcinoma. *Gut* 2012; **61**: 427-438 [PMID: 21930732 DOI: 10.1136/gutjnl-2011-300509]
30 **Li YW**, Qiu SJ, Fan J, Zhou J, Gao Q, Xiao YS, Xu YF. Intratumoral neutrophils: a poor prognostic factor for hepatocellular carcinoma following resection. *J Hepatol* 2011; **54**: 497-505 [PMID: 21112656 DOI: 10.1016/j.jhep.2010.07.044]
31 **Hoffmann JA**, Kafatos FC, Janeway CA, Ezekowitz RA. Phylogenetic perspectives in innate immunity. *Science* 1999; **284**: 1313-1318 [PMID: 10334979 DOI: 10.1126/science.284.5418.1313]
32 **Akira S**, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol* 2004; **4**: 499-511 [PMID: 15229469 DOI: 10.1038/nri1391]
33 **Fearon DT**. Seeking wisdom in innate immunity. *Nature* 1997; **388**: 323-324 [PMID: 9237746 DOI: 10.1038/40967]
34 **Creagh EM**, O'Neill LA. TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. *Trends Immunol* 2006; **27**: 352-357 [PMID: 16807108 DOI: 10.1016/j.it.2006.06.003]
35 **Hashimoto C**, Hudson KL, Anderson KV. The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein. *Cell* 1988; **52**: 269-279 [PMID: 2449285 DOI: 10.1016/0092-8674(88)90516-8]
36 **Lemaitre B**, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* 1996; **86**: 973-983 [PMID: 8808632 DOI: 10.1016/S0092-8674(00)80172-5]
37 **Medzhitov R**, Preston-Hurlburt P, Janeway CA. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 1997; **388**: 394-397 [PMID: 9237759 DOI: 10.1038/41131]
38 **Kawai T**, Akira S. TLR signaling. *Cell Death Differ* 2006; **13**: 816-825 [PMID: 16410796 DOI: 10.1038/sj.cdd.4401850]
39 **Akira S**, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; **124**: 783-801 [PMID: 16497588 DOI: 10.1016/j.cell.2006.02.015]
40 **Coll RC**, O'Neill LA. New insights into the regulation of signalling by toll-like receptors and nod-like receptors. *J Innate Immun* 2010; **2**: 406-421 [PMID: 20505309 DOI: 10.1159/000315469]
41 **Roach JC**, Glusman G, Rowen L, Kaur A, Purcell MK, Smith KD, Hood LE, Aderem A. The evolution of vertebrate Toll-like receptors. *Proc Natl Acad Sci USA* 2005; **102**: 9577-9582 [PMID: 15976025 DOI: 10.1073/pnas.0502272102]
42 **Tsujita T**, Tsukada H, Nakao M, Oshiumi H, Matsumoto M, Seya T. Sensing bacterial flagellin by membrane and soluble orthologs of Toll-like receptor 5 in rainbow trout (Oncorhynchus mikiss). *J Biol Chem* 2004; **279**: 48588-48597 [PMID: 15339910 DOI: 10.1074/jbc.M407634200]
43 **Henneke P**, Takeuchi O, van Strijp JA, Guttormsen HK, Smith JA, Schromm AB, Espevik TA, Akira S, Nizet V, Kasper DL, Golenbock DT. Novel engagement of CD14 and multiple toll-like receptors by group B streptococci. *J Immunol* 2001; **167**: 7069-7076 [PMID: 11739528]
44 **Zarember KA**, Godowski PJ. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J Immunol* 2002; **168**: 554-561 [PMID: 11777946]
45 **Schwabe RF**, Seki E, Brenner DA. Toll-like receptor signaling in the liver. *Gastroenterology* 2006; **130**: 1886-1900 [PMID: 16697751 DOI: 10.1053/j.gastro.2006.01.038]
46 **Szabo G**, Dolganiuc A, Mandrekar P. Pattern recognition receptors: a contemporary view on liver diseases. *Hepatology* 2006; **44**: 287-298 [PMID: 16871558 DOI: 10.1002/hep.21308]
47 **Liu S**, Gallo DJ, Green AM, Williams DL, Gong X, Shapiro RA, Gambotto AA, Humphris EL, Vodovotz Y, Billiar TR. Role of toll-like receptors in changes in gene expression and NF-kappa B activation in mouse hepatocytes stimulated with lipopolysaccharide. *Infect Immun* 2002; **70**: 3433-3442 [PMID: 12065483 DOI: 10.1128/IAI.70.7.3433-3442.2002]
48 **Matsumura T**, Ito A, Takii T, Hayashi H, Onozaki K. Endotoxin and cytokine regulation of toll-like receptor (TLR) 2 and TLR4 gene expression in murine liver and hepatocytes. *J Interferon Cytokine Res* 2000; **20**: 915-921 [PMID: 11054280 DOI: 10.1089/10799900050163299]
49 **Wu J**, Meng Z, Jiang M, Zhang E, Trippler M, Broering R, Bucchi A, Krux F, Dittmer U, Yang D, Roggendorf M, Gerken G, Lu M, Schlaak JF. Toll-like receptor-induced innate immune responses in non-parenchymal liver cells are cell type-specific. *Immunology* 2010; **129**: 363-374 [PMID: 19922426 DOI: 10.1111/j.1365-2567.2009.03179.x]
50 **Martin-Armas M**, Simon-Santamaria J, Pettersen I, Moens U, Smedsrød B, Sveinbjørnsson B. Toll-like receptor 9 (TLR9) is present in murine liver sinusoidal endothelial cells (LSECs) and mediates the effect of CpG-oligonucleotides. *J Hepatol* 2006; **44**: 939-946 [PMID: 16458386 DOI: 10.1016/j.jhep.2005.09.020]
51 **Su GL**, Klein RD, Aminlari A, Zhang HY, Steinstraesser L, Alarcon WH, Remick DG, Wang SC. Kupffer cell activation

by lipopolysaccharide in rats: role for lipopolysaccharide binding protein and toll-like receptor 4. *Hepatology* 2000; **31**: 932-936 [PMID: 10733550 DOI: 10.1053/he.2000.5634]
52 **Thobe BM**, Frink M, Hildebrand F, Schwacha MG, Hubbard WJ, Choudhry MA, Chaudry IH. The role of MAPK in Kupffer cell toll-like receptor (TLR) 2-, TLR4-, and TLR9-mediated signaling following trauma-hemorrhage. *J Cell Physiol* 2007; **210**: 667-675 [PMID: 17117477]
53 **Paik YH**, Schwabe RF, Bataller R, Russo MP, Jobin C, Brenner DA. Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells. *Hepatology* 2003; **37**: 1043-1055 [PMID: 12717385 DOI: 10.1053/jhep.2003.50182]
54 **Seki E**, De Minicis S, Osterreicher CH, Kluwe J, Osawa Y, Brenner DA, Schwabe RF. TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat Med* 2007; **13**: 1324-1332 [PMID: 17952090 DOI: 10.1038/nm1663]
55 **Watanabe A**, Hashmi A, Gomes DA, Town T, Badou A, Flavell RA, Mehal WZ. Apoptotic hepatocyte DNA inhibits hepatic stellate cell chemotaxis via toll-like receptor 9. *Hepatology* 2007; **46**: 1509-1518 [PMID: 17705260 DOI: 10.1002/hep.21867]
56 **Hsu W**, Shu SA, Gershwin E, Lian ZX. The current immune function of hepatic dendritic cells. *Cell Mol Immunol* 2007; **4**: 321-328 [PMID: 17976311]
57 **Edwards AD**, Diebold SS, Slack EM, Tomizawa H, Hemmi H, Kaisho T, Akira S, Reis e Sousa C. Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines. *Eur J Immunol* 2003; **33**: 827-833 [PMID: 12672047 DOI: 10.1002/eji.200323797]
58 **Ma F**, Zhang J, Zhang J, Zhang C. The TLR7 agonists imiquimod and gardiquimod improve DC-based immunotherapy for melanoma in mice. *Cell Mol Immunol* 2010; **7**: 381-388 [PMID: 20543857 DOI: 10.1038/cmi.2010.30]
59 **Chen Y**, Sun R. Toll-like receptors in acute liver injury and regeneration. *Int Immunopharmacol* 2011; **11**: 1433-1441 [PMID: 21601014 DOI: 10.1016/j.intimp.2011.04.023]
60 **Xu D**, Liu H, Komai-Koma M. Direct and indirect role of Toll-like receptors in T cell mediated immunity. *Cell Mol Immunol* 2004; **1**: 239-246 [PMID: 16225766]
61 **Sawaki J**, Tsutsui H, Hayashi N, Yasuda K, Akira S, Tanizawa T, Nakanishi K. Type 1 cytokine/chemokine production by mouse NK cells following activation of their TLR/MyD88-mediated pathways. *Int Immunol* 2007; **19**: 311-320 [PMID: 17289654 DOI: 10.1093/intimm/dxl148]
62 **Meyer-Bahlburg A**, Khim S, Rawlings DJ. B cell intrinsic TLR signals amplify but are not required for humoral immunity. *J Exp Med* 2007; **204**: 3095-3101 [PMID: 18039950 DOI: 10.1084/jem.20071250]
63 **Gelman AE**, Zhang J, Choi Y, Turka LA. Toll-like receptor ligands directly promote activated CD4+ T cell survival. *J Immunol* 2004; **172**: 6065-6073 [PMID: 15128790]
64 **Komai-Koma M**, Jones L, Ogg GS, Xu D, Liew FY. TLR2 is expressed on activated T cells as a costimulatory receptor. *Proc Natl Acad Sci USA* 2004; **101**: 3029-3034 [PMID: 14981245 DOI: 10.1073/pnas.0400171101]
65 **Qian F**, Bolen CR, Jing C, Wang X, Zheng W, Zhao H, Fikrig E, Bruce RD, Kleinstein SH, Montgomery RR. Impaired toll-like receptor 3-mediated immune responses from macrophages of patients chronically infected with hepatitis C virus. *Clin Vaccine Immunol* 2013; **20**: 146-155 [PMID: 23220997 DOI: 10.1128/CVI.00530-12]
66 **Chisari FV**, Isogawa M, Wieland SF. Pathogenesis of hepatitis B virus infection. *Pathol Biol* (Paris) 2010; **58**: 258-266 [PMID: 20116937 DOI: 10.1016/j.patbio.2009.11.001]
67 **Li YJ**, Zhu P, Liang Y, Yin WG, Xiao JH. Hepatitis B virus induces expression of cholesterol metabolism-related genes via TLR2 in HepG2 cells. *World J Gastroenterol* 2013; **19**: 2262-2269 [PMID: 23599654 DOI: 10.3748/wjg.v19.i14.2262]
68 **Chen Y**, Chen Y, Zhao L, Chen Y, Mei M, Li Q, Huang A, Varghese Z, Moorhead JF, Ruan XZ. Inflammatory stress exacerbates hepatic cholesterol accumulation via disrupting cellular cholesterol export. *J Gastroenterol Hepatol* 2012; **27**: 974-984 [PMID: 22098164 DOI: 10.1111/j.1440-1746.2011.06986.x]
69 **Isogawa M**, Robek MD, Furuichi Y, Chisari FV. Toll-like receptor signaling inhibits hepatitis B virus replication in vivo. *J Virol* 2005; **79**: 7269-7272 [PMID: 15890966 DOI: 10.1128/JVI.79.11.7269-7272.2005]
70 **Dolganiuc A**, Oak S, Kodys K, Golenbock DT, Finberg RW, Kurt-Jones E, Szabo G. Hepatitis C core and nonstructural 3 proteins trigger toll-like receptor 2-mediated pathways and inflammatory activation. *Gastroenterology* 2004; **127**: 1513-1524 [PMID: 15521019 DOI: 10.1053/j.gastro.2004.08.067]
71 **Machida K**, Cheng KT, Sung VM, Levine AM, Foung S, Lai MM. Hepatitis C virus induces toll-like receptor 4 expression, leading to enhanced production of beta interferon and interleukin-6. *J Virol* 2006; **80**: 866-874 [PMID: 16378988 DOI: 10.1128/JVI.80.2.866-874.2006]
72 **Hosomura N**, Kono H, Tsuchiya M, Ishii K, Ogiku M, Matsuda M, Fujii H. HCV-related proteins activate Kupffer cells isolated from human liver tissues. *Dig Dis Sci* 2011; **56**: 1057-1064 [PMID: 20848204 DOI: 10.1007/s10620-010-1395-y]
73 **Wang JP**, Zhang Y, Wei X, Li J, Nan XP, Yu HT, Li Y, Wang PZ, Bai XF. Circulating Toll-like receptor (TLR) 2, TLR4, and regulatory T cells in patients with chronic hepatitis C. *APMIS* 2010; **118**: 261-270 [PMID: 20402671 DOI: 10.1111/j.1600-0463.2010.02586.x]
74 **Zhang Y**, Lian JQ, Huang CX, Wang JP, Wei X, Nan XP, Yu HT, Jiang LL, Wang XQ, Zhuang Y, Li XH, Li Y, Wang PZ, Robek MD, Bai XF. Overexpression of Toll-like receptor 2/4 on monocytes modulates the activities of CD4(+)CD25(+) regulatory T cells in chronic hepatitis B virus infection. *Virology* 2010; **397**: 34-42 [PMID: 19945134 DOI: 10.1016/j.virol.2009.11.007]
75 **Berzsenyi MD**, Roberts SK, Preiss S, Woollard DJ, Beard MR, Skinner NA, Bowden DS, Visvanathan K. Hepatic TLR2 & TLR4 expression correlates with hepatic inflammation and TNF-α in HCV & HCV/HIV infection. *J Viral Hepat* 2011; **18**: 852-860 [PMID: 21050341 DOI: 10.1111/j.1365-2893.2010.01390.x]
76 **Heim MH**. Innate immunity and HCV. *J Hepatol* 2013; **58**: 564-574 [PMID: 23063572 DOI: 10.1016/j.jhep.2012.10.005]
77 **Imran M**, Waheed Y, Manzoor S, Bilal M, Ashraf W, Ali M, Ashraf M. Interaction of Hepatitis C virus proteins with pattern recognition receptors. *Virol J* 2012; **9**: 126 [PMID: 22726246 DOI: 10.1186/1743-422X-9-126]
78 **Sawhney R**, Visvanathan K. Polymorphisms of toll-like receptors and their pathways in viral hepatitis. *Antivir Ther* 2011; **16**: 443-458 [PMID: 21685532 DOI: 10.3851/IMP1820]
79 **Gao B**, Bataller R. Alcoholic liver disease: pathogenesis and new therapeutic targets. *Gastroenterology* 2011; **141**: 1572-1585 [PMID: 21920463 DOI: 10.1053/j.gastro.2011.09.002]
80 **Purohit V**, Gao B, Song BJ. Molecular mechanisms of alcoholic fatty liver. *Alcohol Clin Exp Res* 2009; **33**: 191-205 [PMID: 19032584 DOI: 10.1111/j.1530-0277.2008.00827.x]
81 **Ajakaiye M**, Jacob A, Wu R, Nicastro JM, Coppa GF, Wang P. Alcohol and hepatocyte-Kupffer cell interaction (review). *Mol Med Rep* 2011; **4**: 597-602 [PMID: 21468548]
82 **Li Z**, Diehl AM. Innate immunity in the liver. *Curr Opin Gastroenterol* 2003; **19**: 565-571 [PMID: 15703606 DOI: 10.1097/00001574-200311000-00009]
83 **Hritz I**, Mandrekar P, Velayudham A, Catalano D, Dolganiuc A, Kodys K, Kurt-Jones E, Szabo G. The critical role of toll-like receptor (TLR) 4 in alcoholic liver disease is independent of the common TLR adapter MyD88. *Hepatology* 2008; **48**: 1224-1231 [PMID: 18792393 DOI: 10.1002/

hep.22470]
84 **Petrasek J**, Dolganiuc A, Csak T, Nath B, Hritz I, Kodys K, Catalano D, Kurt-Jones E, Mandrekar P, Szabo G. Interferon regulatory factor 3 and type I interferons are protective in alcoholic liver injury in mice by way of crosstalk of parenchymal and myeloid cells. *Hepatology* 2011; **53**: 649-660 [PMID: 21274885 DOI: 10.1002/hep.24059]
85 **Miura K**, Kodama Y, Inokuchi S, Schnabl B, Aoyama T, Ohnishi H, Olefsky JM, Brenner DA, Seki E. Toll-like receptor 9 promotes steatohepatitis by induction of interleukin-1beta in mice. *Gastroenterology* 2010; **139**: 323-34.e7 [PMID: 20347818 DOI: 10.1053/j.gastro.2010.03.052]
86 **Rao R**. Endotoxemia and gut barrier dysfunction in alcoholic liver disease. *Hepatology* 2009; **50**: 638-644 [PMID: 19575462 DOI: 10.1002/hep.23009]
87 **Yin M**, Wheeler MD, Kono H, Bradford BU, Gallucci RM, Luster MI, Thurman RG. Essential role of tumor necrosis factor alpha in alcohol-induced liver injury in mice. *Gastroenterology* 1999; **117**: 942-952 [PMID: 10500078 DOI: 10.1016/S0016-5085(99)70354-9]
88 **Machida K**, Tsukamoto H, Mkrtchyan H, Duan L, Dynnyk A, Liu HM, Asahina K, Govindarajan S, Ray R, Ou JH, Seki E, Deshaies R, Miyake K, Lai MM. Toll-like receptor 4 mediates synergism between alcohol and HCV in hepatic oncogenesis involving stem cell marker Nanog. *Proc Natl Acad Sci USA* 2009; **106**: 1548-1553 [PMID: 19171902 DOI: 10.1073/pnas.0807390106]
89 **Pritchard MT**, McMullen MR, Stavitsky AB, Cohen JI, Lin F, Medof ME, Nagy LE. Differential contributions of C3, C5, and decay-accelerating factor to ethanol-induced fatty liver in mice. *Gastroenterology* 2007; **132**: 1117-1126 [PMID: 17383432 DOI: 10.1053/j.gastro.2007.01.053]
90 **Cohen JI**, Roychowdhury S, McMullen MR, Stavitsky AB, Nagy LE. Complement and alcoholic liver disease: role of C1q in the pathogenesis of ethanol-induced liver injury in mice. *Gastroenterology* 2010; **139**: 664-74, 674.e1 [PMID: 20416309 DOI: 10.1053/j.gastro.2010.04.041]
91 **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]
92 **Cohen JC**, Horton JD, Hobbs HH. Human fatty liver disease: old questions and new insights. *Science* 2011; **332**: 1519-1523 [PMID: 21700865 DOI: 10.1126/science.1204265]
93 **Farrell GC**, van Rooyen D, Gan L, Chitturi S. NASH is an Inflammatory Disorder: Pathogenic, Prognostic and Therapeutic Implications. *Gut Liver* 2012; **6**: 149-171 [PMID: 22570745 DOI: 10.5009/gnl.2012.6.2.149]
94 **Farrell GC**, Larter CZ. Nonalcoholic fatty liver disease: from steatosis to cirrhosis. *Hepatology* 2006; **43**: S99-S112 [PMID: 16447287]
95 **Valenti L**, Fracanzani AL, Fargion S. The immunopathogenesis of alcoholic and nonalcoholic steatohepatitis: two triggers for one disease? *Semin Immunopathol* 2009; **31**: 359-369 [PMID: 19440711 DOI: 10.1007/s00281-009-0152-9]
96 **Petrasek J**, Csak T, Ganz M, Szabo G. Differences in innate immune signaling between alcoholic and non-alcoholic steatohepatitis. *J Gastroenterol Hepatol* 2013; **28** Suppl 1: 93-98 [PMID: 23855302 DOI: 10.1111/jgh.12020]
97 **Csak T**, Dolganiuc A, Kodys K, Nath B, Petrasek J, Bala S, Lippai D, Szabo G. Mitochondrial antiviral signaling protein defect links impaired antiviral response and liver injury in steatohepatitis in mice. *Hepatology* 2011; **53**: 1917-1931 [PMID: 21425308 DOI: 10.1002/hep.24301]
98 **Rivera CA**, Adegboyega P, van Rooijen N, Tagalicud A, Allman M, Wallace M. Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis. *J Hepatol* 2007; **47**: 571-579 [PMID: 17644211 DOI: 10.1016/j.jhep.2007.04.019]
99 **Miura K**, Yang L, van Rooijen N, Ohnishi H, Seki E. Hepatic recruitment of macrophages promotes nonalcoholic steatohepatitis through CCR2. *Am J Physiol Gastrointest Liver Physiol* 2012; **302**: G1310-G1321 [PMID: 22442158 DOI: 10.1152/ajpgi.00365.2011]
100 **Adachi Y**, Bradford BU, Gao W, Bojes HK, Thurman RG. Inactivation of Kupffer cells prevents early alcohol-induced liver injury. *Hepatology* 1994; **20**: 453-460 [PMID: 8045507 DOI: 10.1002/hep.1840200227]
101 **Bertola A**, Bonnafous S, Anty R, Patouraux S, Saint-Paul MC, Iannelli A, Gugenheim J, Barr J, Mato JM, Le March-and-Brustel Y, Tran A, Gual P. Hepatic expression patterns of inflammatory and immune response genes associated with obesity and NASH in morbidly obese patients. *PLoS One* 2010; **5**: e13577 [PMID: 21042596 DOI: 10.1371/journal.pone.0013577]
102 **Broering R**, Lu M, Schlaak JF. Role of Toll-like receptors in liver health and disease. *Clin Sci* (Lond) 2011; **121**: 415-426 [PMID: 21797822 DOI: 10.1042/CS20110065]
103 **Wagnerberger S**, Spruss A, Kanuri G, Volynets V, Stahl C, Bischoff SC, Bergheim I. Toll-like receptors 1-9 are elevated in livers with fructose-induced hepatic steatosis. *Br J Nutr* 2012; **107**: 1727-1738 [PMID: 22018861 DOI: 10.1017/S0007114511004983]
104 **Csak T**, Velayudham A, Hritz I, Petrasek J, Levin I, Lippai D, Catalano D, Mandrekar P, Dolganiuc A, Kurt-Jones E, Szabo G. Deficiency in myeloid differentiation factor-2 and toll-like receptor 4 expression attenuates nonalcoholic steatohepatitis and fibrosis in mice. *Am J Physiol Gastrointest Liver Physiol* 2011; **300**: G433-G441 [PMID: 21233280 DOI: 10.1152/ajpgi.00163.2009]
105 **Szabo G**, Velayudham A, Romics L, Mandrekar P. Modulation of non-alcoholic steatohepatitis by pattern recognition receptors in mice: the role of toll-like receptors 2 and 4. *Alcohol Clin Exp Res* 2005; **29**(11 Suppl): 140S-145S [PMID: 16344599 DOI: 10.1097/01.alc.0000189287.83544.33]
106 **Maeda S**. NF-κB, JNK, and TLR Signaling Pathways in Hepatocarcinogenesis. *Gastroenterol Res Pract* 2010; **2010**: 367694 [PMID: 21151655 DOI: 10.1155/2010/367694]
107 **Szlosarek PW**, Balkwill FR. Tumour necrosis factor alpha: a potential target for the therapy of solid tumours. *Lancet Oncol* 2003; **4**: 565-573 [PMID: 12965278 DOI: 10.1016/S1470-2045(03)01196-3]
108 **Sakurai T**, Maeda S, Chang L, Karin M. Loss of hepatic NF-kappa B activity enhances chemical hepatocarcinogenesis through sustained c-Jun N-terminal kinase 1 activation. *Proc Natl Acad Sci USA* 2006; **103**: 10544-10551 [PMID: 16807293 DOI: 10.1073/pnas.0603499103]
109 **Kamata H**, Honda S, Maeda S, Chang L, Hirata H, Karin M. Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell* 2005; **120**: 649-661 [PMID: 15766528 DOI: 10.1016/j.cell.2004.12.041]
110 **Maeda S**, Kamata H, Luo JL, Leffert H, Karin M. IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. *Cell* 2005; **121**: 977-990 [PMID: 15989949 DOI: 10.1016/j.cell.2005.04.014]
111 **Luedde T**, Beraza N, Kotsikoris V, van Loo G, Nenci A, De Vos R, Roskams T, Trautwein C, Pasparakis M. Deletion of NEMO/IKKgamma in liver parenchymal cells causes steatohepatitis and hepatocellular carcinoma. *Cancer Cell* 2007; **11**: 119-132 [PMID: 17292824 DOI: 10.1016/j.ccr.2006.12.016]
112 **Inokuchi S**, Aoyama T, Miura K, Osterreicher CH, Kodama Y, Miyai K, Akira S, Brenner DA, Seki E. Disruption of TAK1 in hepatocytes causes hepatic injury, inflammation, fibrosis, and carcinogenesis. *Proc Natl Acad Sci USA* 2010; **107**: 844-849 [PMID: 20080763 DOI: 10.1073/pnas.0909781107]
113 **Nischalke HD**, Coenen M, Berger C, Aldenhoff K, Müller T, Berg T, Krämer B, Körner C, Odenthal M, Schulze F, Grünhage F, Nattermann J, Sauerbruch T, Spengler U. The toll-

like receptor 2 (TLR2) -196 to -174 del/ins polymorphism affects viral loads and susceptibility to hepatocellular carcinoma in chronic hepatitis C. *Int J Cancer* 2012; **130**: 1470-1475 [PMID: 21500195 DOI: 10.1002/ijc.26143]
114 **Kutikhin AG**. Association of polymorphisms in TLR genes and in genes of the Toll-like receptor signaling pathway with cancer risk. *Hum Immunol* 2011; **72**: 1095-1116 [PMID: 21872627 DOI: 10.1016/j.humimm.2011.07.307]
115 **Zhang H**, Zhai Y, Hu Z, Wu C, Qian J, Jia W, Ma F, Huang W, Yu L, Yue W, Wang Z, Li P, Zhang Y, Liang R, Wei Z, Cui Y, Xie W, Cai M, Yu X, Yuan Y, Xia X, Zhang X, Yang H, Qiu W, Yang J, Gong F, Chen M, Shen H, Lin D, Zeng YX, He F, Zhou G. Genome-wide association study identifies 1p36.22 as a new susceptibility locus for hepatocellular carcinoma in chronic hepatitis B virus carriers. *Nat Genet* 2010; **42**: 755-758 [PMID: 20676096 DOI: 10.1038/ng.638]
116 **Junjie X**, Songyao J, Minmin S, Yanyan S, Baiyong S, Xiaxing D, Jiabin J, Xi Z, Hao C. The association between Toll-like receptor 2 single-nucleotide polymorphisms and hepatocellular carcinoma susceptibility. *BMC Cancer* 2012; **12**: 57 [PMID: 22309608 DOI: 10.1186/1471-2407-12-57]
117 **Fujita Y**, Taguchi H. Overview and outlook of Toll-like receptor ligand-antigen conjugate vaccines. *Ther Deliv* 2012; **3**: 749-760 [PMID: 22838070 DOI: 10.4155/tde.12.52]
118 **Weeratna RD**, Makinen SR, McCluskie MJ, Davis HL. TLR agonists as vaccine adjuvants: comparison of CpG ODN and Resiquimod (R-848). *Vaccine* 2005; **23**: 5263-5270 [PMID: 16081189 DOI: 10.1016/j.vaccine.2005.06.024]
119 **Hoebe K**, Jiang Z, Georgel P, Tabeta K, Janssen E, Du X, Beutler B. TLR signaling pathways: opportunities for activation and blockade in pursuit of therapy. *Curr Pharm Des* 2006; **12**: 4123-4134 [PMID: 17100615 DOI: 10.2174/138161206778743466]
120 **Vasileiou I**, Kostopanagiotou G, Katsargyris A, Klonaris C, Perrea D, Theocharis S. Toll-like receptors: a novel target for therapeutic intervention in intestinal and hepatic ischemia-reperfusion injury? *Expert Opin Ther Targets* 2010; **14**: 839-853 [PMID: 20568914 DOI: 10.1517/14728222.2010.500286]
121 **Kanzler H**, Barrat FJ, Hessel EM, Coffman RL. Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. *Nat Med* 2007; **13**: 552-559 [PMID: 17479101 DOI: 10.1038/nm1589]
122 **Thompson AJ**, Colledge D, Rodgers S, Wilson R, Revill P, Desmond P, Mansell A, Visvanathan K, Locarnini S. Stimulation of the interleukin-1 receptor and Toll-like receptor 2 inhibits hepatitis B virus replication in hepatoma cell lines in vitro. *Antivir Ther* 2009; **14**: 797-808 [PMID: 19812442 DOI: 10.3851/IMP1294]
123 **Horsmans Y**, Berg T, Desager JP, Mueller T, Schott E, Fletcher SP, Steffy KR, Bauman LA, Kerr BM, Averett DR. Isatoribine, an agonist of TLR7, reduces plasma virus concentration in chronic hepatitis C infection. *Hepatology* 2005; **42**: 724-731 [PMID: 16116638 DOI: 10.1002/hep.20839]
124 **Ma R**, Du JL, Huang J, Wu CY. Additive effects of CpG ODN and R-848 as adjuvants on augmenting immune responses to HBsAg vaccination. *Biochem Biophys Res Commun* 2007; **361**: 537-542 [PMID: 17662694 DOI: 10.1016/j.bbrc.2007.07.028]
125 **Gupta K**, Cooper C. A review of the role of CpG oligodeoxynucleotides as toll-like receptor 9 agonists in prophylactic and therapeutic vaccine development in infectious diseases. *Drugs R D* 2008; **9**: 137-145 [PMID: 18457466 DOI: 10.2165/00126839-200809030-00001]
126 **Sung JJ**, Lik-Yuen H. HBV-ISS (Dynavax). *Curr Opin Mol Ther* 2006; **8**: 150-155 [PMID: 16610768]
127 **Cooper CL**, Davis HL, Morris ML, Efler SM, Adhami MA, Krieg AM, Cameron DW, Heathcote J. CPG 7909, an immunostimulatory TLR9 agonist oligodeoxynucleotide, as adjuvant to Engerix-B HBV vaccine in healthy adults: a double-blind phase I/II study. *J Clin Immunol* 2004; **24**: 693-701 [PMID: 15622454 DOI: 10.1007/s10875-004-6244-3]
128 **Rossignol DP**, Lynn M. Antagonism of in vivo and ex vivo response to endotoxin by E5564, a synthetic lipid A analogue. *J Endotoxin Res* 2002; **8**: 483-488 [PMID: 12697095 DOI: 10.1179/096805102125001127]
129 **Kawamoto T**, Ii M, Kitazaki T, Iizawa Y, Kimura H. TAK-242 selectively suppresses Toll-like receptor 4-signaling mediated by the intracellular domain. *Eur J Pharmacol* 2008; **584**: 40-48 [PMID: 18299127 DOI: 10.1016/j.ejphar.2008.01.026]
130 **Cowden JM**, Yu F, Challapalli M, Huang JF, Kim S, Fung-Leung WP, Ma JY, Riley JP, Zhang M, Dunford PJ, Thurmond RL. Antagonism of the histamine H4 receptor reduces LPS-induced TNF production in vivo. *Inflamm Res* 2013; **62**: 599-607 [PMID: 23532396 DOI: 10.1007/s00011-013-0612-5]
131 **Loiarro M**, Ruggiero V, Sette C. Targeting the Toll-like receptor/interleukin 1 receptor pathway in human diseases: rational design of MyD88 inhibitors. *Clin Lymphoma Myeloma Leuk* 2013; **13**: 222-226 [PMID: 23490990 DOI: 10.1016/j.clml.2013.02.003]
132 **Bartfai T**, Behrens MM, Gaidarova S, Pemberton J, Shivanyuk A, Rebek J Jr. A low molecular weight mimic of the Toll/IL-1 receptor/resistance domain inhibits IL-1 receptor-mediated responses. *Proc Natl Acad Sci USA* 2003; **100**: 7971-7976 [PMID: 12799462 DOI: 10.1073/pnas.0932746100]
133 **Loiarro M**, Capolunghi F, Fantò N, Gallo G, Campo S, Arseni B, Carsetti R, Carminati P, De Santis R, Ruggiero V, Sette C. Pivotal Advance: Inhibition of MyD88 dimerization and recruitment of IRAK1 and IRAK4 by a novel peptidomimetic compound. *J Leukoc Biol* 2007; **82**: 801-810 [PMID: 17548806 DOI: 10.1189/jlb.1206746]
134 **Song KW**, Talamas FX, Suttmann RT, Olson PS, Barnett JW, Lee SW, Thompson KD, Jin S, Hekmat-Nejad M, Cai TZ, Manning AM, Hill RJ, Wong BR. The kinase activities of interleukin-1 receptor associated kinase (IRAK)-1 and 4 are redundant in the control of inflammatory cytokine expression in human cells. *Mol Immunol* 2009; **46**: 1458-1466 [PMID: 19181383 DOI: 10.1016/j.molimm.2008.12.012]
135 **Aravalli RN**. Development of microRNA therapeutics for hepatocellular carcinoma. *Diagnostics* 2013; **3**: 170-191 [DOI: 10.3390/diagnostics3010170]
136 **Gramantieri L**, Fornari F, Callegari E, Sabbioni S, Lanza G, Croce CM, Bolondi L, Negrini M. MicroRNA involvement in hepatocellular carcinoma. *J Cell Mol Med* 2008; **12**: 2189-2204 [PMID: 19120703 DOI: 10.1111/j.1582-4934.2008.00533.x]
137 **Ura S**, Honda M, Yamashita T, Ueda T, Takatori H, Nishino R, Sunakozaka H, Sakai Y, Horimoto K, Kaneko S. Differential microRNA expression between hepatitis B and hepatitis C leading disease progression to hepatocellular carcinoma. *Hepatology* 2009; **49**: 1098-1112 [PMID: 19173277 DOI: 10.1002/hep.22749]
138 **Murakami Y**, Yasuda T, Saigo K, Urashima T, Toyoda H, Okanoue T, Shimotohno K. Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. *Oncogene* 2006; **25**: 2537-2545 [PMID: 16331254 DOI: 10.1038/sj.onc.1209283]
139 **Huang S**, He X. The role of microRNAs in liver cancer progression. *Br J Cancer* 2011; **104**: 235-240 [PMID: 21102580 DOI: 10.1038/sj.bjc.6606010]
140 **Budhu A**, Jia HL, Forgues M, Liu CG, Goldstein D, Lam A, Zanetti KA, Ye QH, Qin LX, Croce CM, Tang ZY, Wang XW. Identification of metastasis-related microRNAs in hepatocellular carcinoma. *Hepatology* 2008; **47**: 897-907 [PMID: 18176954 DOI: 10.1002/hep.22160]
141 **Huang YS**, Dai Y, Yu XF, Bao SY, Yin YB, Tang M, Hu CX. Microarray analysis of microRNA expression in hepatocellular carcinoma and non-tumorous tissues without viral hepatitis. *J Gastroenterol Hepatol* 2008; **23**: 87-94 [PMID:

18171346 DOI: 10.1111/j.1440-1746.2007.05223.x]
142 **Jiang J**, Gusev Y, Aderca I, Mettler TA, Nagorney DM, Brackett DJ, Roberts LR, Schmittgen TD. Association of MicroRNA expression in hepatocellular carcinomas with hepatitis infection, cirrhosis, and patient survival. *Clin Cancer Res* 2008; **14**: 419-427 [PMID: 18223217 DOI: 10.1158/1078-0432.CCR-07-0523]
143 **Ladeiro Y**, Couchy G, Balabaud C, Bioulac-Sage P, Pelletier L, Rebouissou S, Zucman-Rossi J. MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. *Hepatology* 2008; **47**: 1955-1963 [PMID: 18433021 DOI: 10.1002/hep.22256]
144 **Li W**, Xie L, He X, Li J, Tu K, Wei L, Wu J, Guo Y, Ma X, Zhang P, Pan Z, Hu X, Zhao Y, Xie H, Jiang G, Chen T, Wang J, Zheng S, Cheng J, Wan D, Yang S, Li Y, Gu J. Diagnostic and prognostic implications of microRNAs in human hepatocellular carcinoma. *Int J Cancer* 2008; **123**: 1616-1622 [PMID: 18649363 DOI: 10.1002/ijc.23693]
145 **Gantier MP**. New perspectives in MicroRNA regulation of innate immunity. *J Interferon Cytokine Res* 2010; **30**: 283-289 [PMID: 20477549 DOI: 10.1089/jir.2010.0037]
146 **Tang B**, Xiao B, Liu Z, Li N, Zhu ED, Li BS, Xie QH, Zhuang Y, Zou QM, Mao XH. Identification of MyD88 as a novel target of miR-155, involved in negative regulation of Helicobacter pylori-induced inflammation. *FEBS Lett* 2010; **584**: 1481-1486 [PMID: 20219467 DOI: 10.1016/j.febslet.2010.02.063]
147 **McCoy CE**, Sheedy FJ, Qualls JE, Doyle SL, Quinn SR, Murray PJ, O'Neill LA. IL-10 inhibits miR-155 induction by toll-like receptors. *J Biol Chem* 2010; **285**: 20492-20498 [PMID: 20435894 DOI: 10.1074/jbc.M110.102111]
148 **Nahid MA**, Satoh M, Chan EK. Mechanistic role of microRNA-146a in endotoxin-induced differential cross-regulation of TLR signaling. *J Immunol* 2011; **186**: 1723-1734 [PMID: 21178010 DOI: 10.4049/jimmunol.1002311]
149 **Nahid MA**, Yao B, Dominguez-Gutierrez PR, Kesavalu L, Satoh M, Chan EK. Regulation of TLR2-mediated tolerance and cross-tolerance through IRAK4 modulation by miR-132 and miR-212. *J Immunol* 2013; **190**: 1250-1263 [PMID: 23264652 DOI: 10.4049/jimmunol.1103060]
150 **El Gazzar M**, Church A, Liu T, McCall CE. MicroRNA-146a regulates both transcription silencing and translation disruption of TNF-α during TLR4-induced gene reprogramming. *J Leukoc Biol* 2011; **90**: 509-519 [PMID: 21562054 DOI: 10.1189/jlb.0211074]
151 **Curtale G**, Mirolo M, Renzi TA, Rossato M, Bazzoni F, Locati M. Negative regulation of Toll-like receptor 4 signaling by IL-10-dependent microRNA-146b. *Proc Natl Acad Sci USA* 2013; **110**: 11499-11504 [PMID: 23798430 DOI: 10.1073/pnas.1219852110]
152 **Taganov KD**, Boldin MP, Chang KJ, Baltimore D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci USA* 2006; **103**: 12481-12486 [PMID: 16885212]
153 **Quinn EM**, Wang JH, O'Callaghan G, Redmond HP. MicroRNA-146a is upregulated by and negatively regulates TLR2 signaling. *PLoS One* 2013; **8**: e62232 [PMID: 23638011 DOI: 10.1371/journal.pone.0062232]
154 **Xie W**, Li Z, Li M, Xu N, Zhang Y. miR-181a and inflammation: miRNA homeostasis response to inflammatory stimuli in vivo. *Biochem Biophys Res Commun* 2013; **430**: 647-652 [PMID: 23220232 DOI: 10.1016/j.bbrc.2012.11.097]
155 **Lai L**, Song Y, Liu Y, Chen Q, Han Q, Chen W, Pan T, Zhang Y, Cao X, Wang Q. MicroRNA-92a negatively regulates Toll-like receptor (TLR)-triggered inflammatory response in macrophages by targeting MKK4 kinase. *J Biol Chem* 2013; **288**: 7956-7967 [PMID: 23355465 DOI: 10.1074/jbc.M112.445429]
156 **Benakanakere MR**, Li Q, Eskan MA, Singh AV, Zhao J, Galicia JC, Stathopoulou P, Knudsen TB, Kinane DF. Modulation of TLR2 protein expression by miR-105 in human oral keratinocytes. *J Biol Chem* 2009; **284**: 23107-23115 [PMID: 19509287 DOI: 10.1074/jbc.M109.013862]
157 **Chen XM**, Splinter PL, O'Hara SP, LaRusso NF. A cellular micro-RNA, let-7i, regulates Toll-like receptor 4 expression and contributes to cholangiocyte immune responses against Cryptosporidium parvum infection. *J Biol Chem* 2007; **282**: 28929-28938 [PMID: 17660297 DOI: 10.1074/jbc.M702633200]
158 **Xue X**, Feng T, Yao S, Wolf KJ, Liu CG, Liu X, Elson CO, Cong Y. Microbiota downregulates dendritic cell expression of miR-10a, which targets IL-12/IL-23p40. *J Immunol* 2011; **187**: 5879-5886 [PMID: 22068236 DOI: 10.4049/jimmunol.1100535]
159 **Liu G**, Friggeri A, Yang Y, Park YJ, Tsuruta Y, Abraham E. miR-147, a microRNA that is induced upon Toll-like receptor stimulation, regulates murine macrophage inflammatory responses. *Proc Natl Acad Sci USA* 2009; **106**: 15819-15824 [PMID: 19721002 DOI: 10.1073/pnas.0901216106]
160 **Wendlandt EB**, Graff JW, Gioannini TL, McCaffrey AP, Wilson ME. The role of microRNAs miR-200b and miR-200c in TLR4 signaling and NF-κB activation. *Innate Immun* 2012; **18**: 846-855 [PMID: 22522429 DOI: 10.1177/1753425912443903]
161 **Pichiorri F**, Suh SS, Ladetto M, Kuehl M, Palumbo T, Drandi D, Taccioli C, Zanesi N, Alder H, Hagan JP, Munker R, Volinia S, Boccadoro M, Garzon R, Palumbo A, Aqeilan RI, Croce CM. MicroRNAs regulate critical genes associated with multiple myeloma pathogenesis. *Proc Natl Acad Sci USA* 2008; **105**: 12885-12890 [PMID: 18728182 DOI: 10.1073/pnas.0806202105]
162 **Sheedy FJ**, Palsson-McDermott E, Hennessy EJ, Martin C, O'Leary JJ, Ruan Q, Johnson DS, Chen Y, O'Neill LA. Negative regulation of TLR4 via targeting of the proinflammatory tumor suppressor PDCD4 by the microRNA miR-21. *Nat Immunol* 2010; **11**: 141-147 [PMID: 19946272 DOI: 10.1038/ni.1828]
163 **Fabbri M**, Paone A, Calore F, Galli R, Gaudio E, Santhanam R, Lovat F, Fadda P, Mao C, Nuovo GJ, Zanesi N, Crawford M, Ozer GH, Wernicke D, Alder H, Caligiuri MA, Nana-Sinkam P, Perrotti D, Croce CM. MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. *Proc Natl Acad Sci USA* 2012; **109**: E2110-E2116 [PMID: 22753494 DOI: 10.1073/pnas.1209414109]
164 **Xu J**, Wu C, Che X, Wang L, Yu D, Zhang T, Huang L, Li H, Tan W, Wang C, Lin D. Circulating microRNAs, miR-21, miR-122, and miR-223, in patients with hepatocellular carcinoma or chronic hepatitis. *Mol Carcinog* 2011; **50**: 136-142 [PMID: 21229610 DOI: 10.1002/mc.20712]
165 **Gailhouste L**, Ochiya T. Cancer-related microRNAs and their role as tumor suppressors and oncogenes in hepatocellular carcinoma. *Histol Histopathol* 2013; **28**: 437-451 [PMID: 23224781]
166 **Qi P**, Cheng SQ, Wang H, Li N, Chen YF, Gao CF. Serum microRNAs as biomarkers for hepatocellular carcinoma in Chinese patients with chronic hepatitis B virus infection. *PLoS One* 2011; **6**: e28486 [PMID: 22174818 DOI: 10.1371/journal.pone.0028486]
167 **Wong KF**, Xu Z, Chen J, Lee NP, Luk JM. Circulating markers for prognosis of hepatocellular carcinoma. *Expert Opin Med Diagn* 2013; **7**: 319-329 [PMID: 23642136 DOI: 10.1517/17530059.2013.795146]
168 **Wilhelm S**, Carter C, Lynch M, Lowinger T, Dumas J, Smith RA, Schwartz B, Simantov R, Kelley S. Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. *Nat Rev Drug Discov* 2006; **5**: 835-844 [PMID: 17016424 DOI: 10.1038/nrd2130]
169 **Kota J**, Chivukula RR, O'Donnell KA, Wentzel EA, Montgomery CL, Hwang HW, Chang TC, Vivekanandan P, Torbenson M, Clark KR, Mendell JR, Mendell JT. Thera-

peutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model. *Cell* 2009; **137**: 1005-1017 [PMID: 19524505 DOI: 10.1016/j.cell.2009.04.021]

170 **Lanford RE**, Hildebrandt-Eriksen ES, Petri A, Persson R, Lindow M, Munk ME, Kauppinen S, Ørum H. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* 2010; **327**: 198-201 [PMID: 19965718 DOI: 10.1126/science.1178178]

171 **Lindow M**, Kauppinen S. Discovering the first microRNA-targeted drug. *J Cell Biol* 2012; **199**: 407-412 [PMID: 23109665 DOI: 10.1083/jcb.201208082]

172 **Bader AG**. miR-34 - a microRNA replacement therapy is headed to the clinic. *Front Genet* 2012; **3**: 120 [PMID: 22783274 DOI: 10.3389/fgene.2012.00120]

P- Reviewers: Cao GW, Gangl A, Morise Z, Wang CC
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.v19.i43.7515

World J Gastroenterol 2013 November 21; 19(43): 7515-7530
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (1): Hepatocellular carcinoma

Bridging and downstaging treatments for hepatocellular carcinoma in patients on the waiting list for liver transplantation

Maurizio Pompili, Giampiero Francica, Francesca Romana Ponziani, Roberto Iezzi, Alfonso Wolfango Avolio

Maurizio Pompili, Francesca Romana Ponziani, Department of Internal Medicine, Università Cattolica del Sacro Cuore, 8-00168 Rome, Italy
Roberto Iezzi, Department of Bioimaging and Radiological Sciences, Università Cattolica del Sacro Cuore, 8-00168 Rome, Italy
Alfonso Wolfango Avolio, Transplant Center, Catholic University, 1-00168 Rome, Italy
Giampiero Francica, Interventional Ultrasound Unit, Pinetagrande Hospital, 81030 Castelvolturno, Italy
Author contributions: Pompili M designed the study, wrote the manuscript, and revised the final version of the article; Francica G, Ponziani FR, Iezzi R and Avolio AW contributed to the literature search and writing the manuscript.
Correspondence to: Maurizio Pompili, MD, Department of Internal Medicine, Università Cattolica del Sacro Cuore, Largo A. Gemelli, 8-00168 Roma, Italy. mpompili@rm.unicatt.it
Telephone: +39-6-30154334 Fax: +39-6-35502775
Received: August 28, 2013 Revised: October 15, 2013
Accepted: October 17, 2013
Published online: November 21, 2013

Abstract

Several therapeutic procedures have been proposed as bridging treatments for patients with hepatocellular carcinoma (HCC) awaiting liver transplantation (LT). The most used treatments include transarterial chemoembolization and radiofrequency ablation. Surgical resection has also been successfully used as a bridging procedure, and LT should be considered a rescue treatment in patients with previous HCC resection who experience tumor recurrence or post-treatment severe decompensation of liver function. The aims of bridging treatments include decreasing the waiting list dropout rate before transplantation, reducing HCC recurrence after transplantation, and improving post-transplant overall survival. To date, no data from prospective randomized studies are available; however, for HCC patients listed for LT within the Milan criteria, prolonging the waiting time over 6-12 mo is a risk factor for tumor spread. Bridging treatments are useful in containing tumor progression and decreasing dropout. Furthermore, the response to pre-LT treatments may represent a surrogate marker of tumor biological aggressiveness and could therefore be evaluated to prioritize HCC candidates for LT. Lastly, although a definitive conclusion can not be reached, the experiences reported to date suggest a positive impact of these treatments on both tumor recurrence and post-transplant patient survival. Advanced HCC may be downstaged to achieve and maintain the current conventional criteria for inclusion in the waiting list for LT. Recent studies have demonstrated that successfully downstaged patients can achieve a 5-year survival rate comparable to that of patients meeting the conventional criteria without requiring downstaging.

Key words: Hepatocellular carcinoma; Bridging treatment; Downstaging; Liver cirrhosis; Liver transplantation; Liver resection; Waiting list; Waiting time; Dropout rate

Core tip: The bridging treatments for patients with hepatocellular carcinoma within Milan criteria listed for liver transplantation are useful in decreasing dropout rate from the waiting list and the experiences reported to date suggest a positive impact on post-transplant tumor recurrence and patient survival. The response to treatments may represent a surrogate marker of tumor biological aggressiveness and could be evaluated to prioritize hepatocellular carcinoma candidates in the waiting list. Advanced hepatocellular carcinoma may be downstaged to achieve the current conventional criteria for inclusion in the waiting list and successfully

downstaged patients can achieve an excellent 5-year survival rate.

Pompili M, Francica G, Ponziani FR, Iezzi R, Avolio AW. Bridging and downstaging treatments for hepatocellular carcinoma in patients on the waiting list for liver transplantation. *World J Gastroenterol* 2013; 19(43): 7515-7530 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7515.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7515

INTRODUCTION

Liver transplantation (LT) is the treatment of choice for patients with unresectable hepatocellular carcinoma (HCC) complicating liver cirrhosis because it allows the cure of both the tumor and the underlying chronic liver disease. HCC classified within the so-called Milan criteria (MC) (1 nodule smaller than 5 cm or no more than 3 nodules smaller than 3 cm)[1] is recognized everywhere as the standard indication for LT. However, after admission to the waiting list for LT, HCC patients can experience tumor growth beyond the conventional transplant criteria. Indeed, there is a high cumulative probability of drop-out from the waiting list for HCC patients due to intrahepatic or extrahepatic tumor progression. This probability has been reported to range between 7% and 11% at 6 mo and to be approximately 38% at 12 mo following enrollment by two papers published at the end of the 1990s by Llovet *et al*[2] and Yao *et al*[3]. The probability has been correlated with tumor characteristics, geographic origin, and length of time waiting for LT[4-6].

Allocation policies for HCC patients awaiting LT remain controversial in the era of the model for end-stage liver disease (MELD) for the management of the LT waiting list. Different models have been developed to quantify the risk of death in neoplastic and non-neoplastic patients[7-11]. As the neoplastic risk assessment is not considered in MELD, patients with unresectable HCC with a neoplasm fulfilling the MC have been considered exceptions in the American allocation system. According to this rule, patients with T2-HCC fulfilling the MC (a single tumor of 2-5 cm or 2-3 tumors each < 3 cm) enter the waiting list with a MELD score equal to 22 and are therefore given priority over patients with less decompensated disease who enter the waiting list according to their laboratory MELD score (6 to 21). In addition, T2-HCC patients also receive incremental points for every 3 mo spent on the waiting list[12,13]. A similar approach has been implemented in other allocation systems[14,15]. The 22 threshold has been set to offer to HCC patients the same drop-out probability of patients without malignancy[16]. More detailed studies based on a dynamic prognosis parallelism have been published, and more complex allocation models aimed at balancing the risk of death in HCC and non-HCC patients have been proposed; however, they have not been applied in clinical practice[7,17-21]. According to these studies, LT candidates with HCC have dropout rates lower than non-HCC candidates, although the rate is similar to that of standard MELD candidates with a score of less than 21. Therefore, HCC patients appear to have an advantage in the current system, raising the question of whether a calculated continuous HCC priority score should be developed that also considers some biological features of the tumor such as the α-fetoprotein (AFP) value, size, and rate of growth[17,18]. Indeed, HCC patients with a high AFP level achieve acceptable LT outcomes if their AFP levels can be reduced with locoregional therapy during the waiting period[22,23]. Furthermore, an inadequate response to HCC bridging therapy was shown to be a strong predictor of dropout probability in three single-center Italian studies[10,14,24], whereas both the serum AFP level and the response to locoregional therapy were related to tumor recurrence and death in a retrospective international multicenter cohort study[25].

Lastly, the development of the survival-benefit approach, which proposes ranking priority according to the benefit in survival between standard care and LT rather than crude survival figures, changed the perspective of the outcome evaluation system[26-28]. The practice most widely used since 2005, which is in accordance with the United Network for Organ Sharing rules, gives HCC patients with unresectable T1 neoplasms (a single nodule smaller than 2 cm) the same priority as patients listed for non-neoplastic diseases.

In this scenario, several therapeutic procedures have been proposed and largely used in the past as bridging neo-adjuvant treatments for patients listed for LT with HCC within the MC[29]. The rationale for their use is the possible decrease of the waiting list drop-out rate before transplantation and of HCC recurrence after transplantation, which is less than 15% in patients with HCC within the MC undergoing LT without any prior tumor treatment[30]. These beneficial effects could also improve the overall survival of transplanted patients. Both surgical resection and locoregional therapies can be used not only as bridging procedures to LT in T2-HCC patients but also to downstage HCC patients who do not initially meet the conventional transplant criteria[31]. According to this approach, patients can be safely listed for LT if they can reach and maintain for an adequate follow-up period the MC or slightly expanded criteria such as the University of California San Francisco criteria (UCSF) (a single HCC ⩽ 6.5 cm or ⩽ 3 tumors with the largest being ⩽ 4.5 cm and a total tumor burden ⩽ 8 cm)[32] or the up-to-7 criteria [HCCs with 7 as the sum of the size of the largest tumor (in cm) and the number of tumors][33]. The aim of downstaging is to select HCC patients with reasonably low rates of tumor recurrence after LT among those who are initially excluded according to the current number-size criteria[34].

In this paper, we analyzed the indications and results of the various neo-adjuvant treatment modalities currently administered to HCC patients awaiting LT to avoid exceeding the MC while on the waiting list as well as those used to downstage patients who do not meet

the conventional transplant criteria.

NEO-ADJUVANT BRIDGING PROCEDURES FOR PATIENTS WITH HEPATOCELLULAR CARCINOMA AWAITING LIVER TRANSPLANTATION

Surgical resection

Liver resection (LR) can be theoretically used as a first-line bridging procedure to LT. However, in most transplant centers, transarterial chemoembolization (TACE) and percutaneous ablation therapies are the preferred bridging therapies. The theoretical advantages of surgery in this setting are twofold. The first advantage is the best possible control of tumor growth, as TACE and percutaneous treatments do not always achieve complete tumor necrosis. The second advantage is the possibility of selecting patients in whom pathological analysis of the resected specimen shows features suggesting poor prognosis in terms of tumor recurrence, such as undifferentiated histotype, satellitosis, microvascular invasion, or capsular effraction, who should immediately undergo evaluation for LT[35]. However, compared to non-surgical therapies, the surgical bridging approach to patients listed for LT implies higher costs, entails more peri-procedural risks, can only be proposed in well-compensated patients without severe portal hypertension, and can make the ensuing LT technically more difficult, with a higher risk of post-operative complications[36].

Moreover, important issues regarding tumor resectability should also be considered. Single exophytic or at least subcapsular neoplasms are easier to resect than multiple neoplasms or those located adjacent to the hilum or vena cava[37,38]. Furthermore, a location in the left lobe represents a more favorable condition, and the progress achieved in the laparoscopic resection of the liver has reduced the number of HCC patients with an absolute indication for LT[39]. However, the combination of LR and LT over time appears to be a reasonable strategy; HCC patients within the MC who have preserved liver function can undergo LR, limiting LT as a rescue treatment in cases of tumor recurrence or liver function failure (salvage LT)[40]. This approach allows a consequent saving of grafts, which can then be more efficaciously transplanted in other patients, and is supported by the discrepancy between the limited donor pool and the enormous number of LT candidates. However, there are important differences in access to LT according to cadaveric organ availability, blood group of the recipient, implementation of a living donor program, and degree of donor-recipient matching[41-44].

Although during the initial experience with LR, the overall survival and disease survival rates of patients undergoing secondary LT after HCC resection were significantly lower (due to higher perioperative mortality unrelated to HCC) than those observed in cirrhotic patients with HCC undergoing primary LT[45], favorable results have more recently been reported by Belghiti *et al*[46]. They showed that postoperative course, complications, and the 3- and 5-year survival rates did not differ significantly between cirrhotic HCC patients undergoing primary LT or secondary LT after resection. Similarly favorable results for salvage LT have been subsequently reported by other groups in patients initially submitted to LR within the MC[15,47] or the UCSF criteria[48].

Salvage LT has been shown to be effective not only in the setting of deceased donor LT but also in the setting of living donor LT, particularly in Asian countries. Compared to deceased donor LT, the main advantage of living donor LT is the reduction of the waiting list time, whereas the main drawback is represented by the occurrence of severe life-threatening complications among donors in approximately 1% of cases[49]. Indeed, Hwang *et al*[50] have shown that the combination of prior recipient hepatectomy and a living donor liver graft is feasible and provides excellent long-term survival in treated patients, and their results have recently been confirmed by other groups[51,52].

Notably, the option of salvage LT cannot be offered to all patients initially treated by LR, primarily due to HCC recurrence overcoming the conventional LT criteria, age over 65 years at the time of recurrence, and the presence of comorbidities preventing the feasibility of LT. In a series reported by Poon *et al*[53], approximately 80% of patients were still eligible for salvage LT at the time of tumor recurrence. In a recent paper by Liu *et al*[48], among 71 patients with HCC recurrence within the UCSF criteria, salvage LT could be performed in 39 patients (54.9%). Compared to 180 HCC patients who underwent primary LT, patients treated with salvage LT for HCC recurrence showed greater intraoperative blood loss and required more blood transfusions; however, perioperative mortality, post-transplant complications, HCC recurrence rates, and overall survival did not differ significantly between the two groups.

Transarterial chemoembolization

TACE is considered the standard treatment for patients with intermediate-stage HCC according to the Barcelona-Clinic Liver Cancer classification[54], and it achieves a partial response in 15%-55% of patients and an improvement of median survival from 16 to 20 mo[55]. The most widely used conventional TACE procedure consists of an arterial infusion of a lipiodol emulsion with a chemotherapeutic agent (*e.g.*, doxorubicin or cisplatin) followed by embolization with gelfoam. However, conventional TACE is not a standardized procedure, and the optimal chemotherapeutic/embolizing agent and retreatment strategy have yet to be determined[56]. In particular, it is well known that TACE requires treatment repetition either at regular intervals or "a la demande" and that repeating conventional TACE may damage non-cancerous hepatocyte functions and affect the clinical course. Indeed, liver toxicity is a major limitation of conventional TACE regimens, and superselective TACE

is recommended in the setting of patients waiting for LT to minimize ischemic injury to non-tumoral liver tissue. Promising new data have been obtained using drug-eluting beads (DEBs), which are particles of variable size that are able to bind and elute doxorubicin in a predictable manner[57]. Compared to conventional techniques, DEBs appear to be a more standardized approach to TACE with less liver-related toxicity and fewer systemic adverse events[58].

TACE has been extensively used in the past as a bridging treatment to LT, and a number of studies have shown that it is an effective therapy in terms of adequate tumor necrosis achievement at explant analysis. Analyzing the largest available series indicates that the rate of patients treated by TACE reaching complete tumor necrosis is quite uniform, ranging between 27% and 57% in patients within the MC[59-67].

Interestingly, the rate of tumor necrosis appears to be higher in patients with single nodules when compared with patients with multiple nodules, in patients submitted to superselective TACE when compared with lobar TACE (complete necrosis achieved in 53.8% *vs* 29.8% of cases, respectively), and in patients with nodules 3-5 cm in size compared with patients with nodules smaller than 3 cm[65]. This last finding confirms the result obtained by Alba *et al*[64] and may be explained considering that larger nodules are typically fed by larger arteries, whereas in some instances, smaller nodules lack fully developed arterial neoangiogenesis; as a result, chemoembolization may be more effective in the former[65]. Accordingly, Kwan *et al*[66] have recently shown that the development of > 90% lesion necrosis upon pathological analysis of explanted liver was associated with avid lesion enhancement and the presence of a feeding vessel larger than 0.9 mm in diameter on the pre-TACE visceral angiogram. On post-TACE computed tomography images, a lack of residual contrast enhancement, a decrease in lesion size, a high lesion density due to an accumulation, and a diffuse distribution of ethiodized oil throughout the lesion were also correlated with near-complete lesion necrosis.

A recent small retrospective study compared tumor response in explanted liver after treatment with DEBs or standard TACE. TACE with DEBs achieved complete necrosis in 77% of the lesions, which was significantly higher than that reached after standard TACE (27.2%). More data are needed to address the better performance of DEBs compared to standard TACE in the transplant setting[68].

Another important point to clarify is the evaluation of TACE safety in patients awaiting LT. Because arteritis of the celiac and hepatic arteries may complicate TACE as a result of endovascular trauma caused by guides and catheters, recipients could be exposed after the transplant to an increased occurrence of complications such as arterial thrombosis. However, the prevalence of such serious complications has not been found to be increased in some studies comparing patients with or without TACE performed before LT[69-71].

Radiofrequency ablation

Radiofrequency thermal ablation (RFA) has gained widespread use over recent years as an effective procedure for small HCCs not amenable to surgical resection. Thermal ablation may be performed using cool-tip or hook needles with comparable results[72]. Some studies have described the use of RFA as a bridge to transplantation in HCC patients in recent years. These studies have reported complete tumor necrosis at pathological evaluation of the explanted liver in 47%-75% of cases, with a mean value of 58%[73-77]. A clear difference in effectiveness can be observed when analyzing tumors according to size. Indeed, the rate of complete necrosis ranges between 50% and 78% in HCCs up to 3 cm and between 13% and 43% in larger neoplasms[73-75,77]. Furthermore, in two studies, a tumor size larger than 3 cm was the only risk factor identified for HCC persistence after treatment[73,75].

Regarding RFA-related complications in the setting of HCC patients awaiting LT, an analysis of the largest available series demonstrated that the procedure is quite safe. In fact, considering 5 large series, the mean rate of post-ablation major complications was only as high as 4.6%, including one case of death due to peritoneal bleeding, two cases of acute peritonitis/cholecystitis, and one case each of severe liver failure treated by urgent transplantation, severe persistent liver failure, biliary stenosis, arterial hemorrhage, and small bowel perforation[73-76,78]. Additionally, the risk of tumor seeding at the level of the abdomen wall appears to be low; however, occasional cases of tumor seeding along the needle track diagnosed after LT in patients submitted to RFA as a bridging procedure have been reported in the literature[79,80].

Other treatments

TACE and RFA are the most used bridging treatments to LT in HCC patients, although other therapeutic options have been proposed (Table 1). Percutaneous ethanol injection (PEI) is the oldest and most used technique for the local treatment of HCC, but it has been rarely used as a bridging treatment to transplantation. In our multicenter survey, the rate of complete necrosis in tumors smaller than 3 cm was 30%[75]. Castroagudín *et al*[81], in a series of 20 nodules in 19 patients, showed that in patients with small tumors (*i.e.*, less than 3 cm), ethanol injection induced complete necrosis in 58% of the cases. In a more recent paper, Branco *et al*[82] reported a complete necrosis rate of 64% in 59 patients within the MC and a mean tumor size of 2.4 cm (range: 0.5-5.5 cm). In these studies, PEI was not affected by procedure-related major complications and did not provide total necrosis in most tumors larger than 3 cm.

Percutaneous laser ablation (PLA) performed using multiple tiny laser fibers has recently been shown to be an effective technique for the thermal ablation of HCC in patients in whom surgical resection is not possible or appropriate[83,84]. We recently showed that in HCC patients awaiting LT, PLA provided results comparable

Table 1 Advantages and disadvantages of the bridging and downstaging procedures for hepatocellular carcinoma in cirrhotic patients who are candidates for liver transplantation

	Advantages	Disadvantages
Resection	Higher complete effectiveness than non-surgical procedures More simple in cases with peripheral subglissonian nodules	Unfeasible in patients with decompensated liver disease or severe portal hypertension
TACE	More effective using the selective/superselective technique in well-vascularized nodules with large feeding arteries	Unfeasible in patients with severely reduced portal vein flow, intratumoral arteriovenous fistula, or renal failure (creatinine clearance < 30 mL/min)
TARE	Possible better effectiveness than TACE in cases with multiple nodules	Less experience with TARE than TACE High cost
RFA	More effective in nodules ≤ 3 cm	Potentially dangerous in patients with impaired clotting parameters or lesions located superficially or near the gallbladder, major bile ducts, or bowel loops
PEI	More effective in nodules ≤ 3 cm Suitable in patients with impaired clotting parameters or lesions located in dangerous sites for thermal ablation	Less effective than RFA for nodules > 2 cm
PLA	More effective in nodules ≤ 3 cm Suitable in patients with impaired clotting parameters	Less experience with PLA than RFA Technically complex Potentially dangerous in cases of lesions located superficially or near the gallbladder, major bile ducts, or bowel loops
MWA	Possible better effectiveness than RFA in nodules ≥ 3 cm or located near large vessels	Less experience with MWA than RFA Potentially dangerous in patients with impaired clotting parameters or with lesions located superficially or near the gallbladder, major bile ducts, or bowel loops

TACE: Transarterial chemoembolization; TARE: Transarterial radio embolization; RFA: Radiofrequency ablation; PEI: Percutaneous ethanol injection; PLA: Percutaneous laser ablation; MWA: Microwave ablation.

to those of RFA; the rate of complete necrosis found at explant analysis in a series of 13 nodules up to 3 cm was 62%[85]. Due to the use of fine needles, the possible advantages of PLA in respect to RFA include the treatment of patients with either nodules in high-risk sites (*i.e.*, near vital structures)[86] or severe clotting impairment, in whom RFA may be contraindicated, and the lower overall cost of the procedure.

Microwave ablation (MWA) has been shown to be an effective thermal ablation procedure for the percutaneous treatment of HCC. Compared to RFA, this technique could theoretically provide a larger volume of necrosis and be more effective when treating nodules adjacent to large vessels; however, a clear advantage of MWA with respect to RFA has not been demonstrated[87,88]. The use of MWA as a bridging procedure to LT or a downstaging procedure in HCC patients appears to be promising. In a recent preliminary study, 6 patients with 6 HCC nodules ranging between 2.5 and 5.0 cm (mean 3.5 cm) in diameter underwent MWA before LT. At explant analysis, all of the nodules showed complete necrosis without intraoperative evidence of tumor spread in all cases or evidence of tumor recurrence at a one-year follow up in the 5 patients who could be evaluated[89].

The effectiveness of transarterial radioembolization (TARE) with 90Yttrium microspheres has recently been evaluated by Riaz *et al*[90], who studied 38 nodules in 35 patients. Of 15 patients with T2-HCC, none progressed to T3-HCC (one nodule > 5 cm or up to three nodules with one > 3 cm) before LT, whereas 8 of 10 patients were downstaged from stage T3 to stage T2. At explant analysis, 23 of the 38 target lesions (61%) showed complete tumor necrosis, and its achievement was affected by the size of the target lesion; indeed, complete necrosis was detected in 89%, 65%, and 33% of lesions smaller than 3 cm, between 3 and 5 cm, and larger than 5 cm, respectively.

Data regarding the use of external conformal radiotherapy (CRT) as a bridging treatment to LT in HCC patients are scarce. In a recent paper, CRT was delivered in five or six fractions to 10 patients with HCC awaiting LT with tumor diameters ranging from 2.5 to 10.8 cm. Nine patients completed the treatment, and it was well tolerated in all cases. Two tumors remained stable; the rest had 10%-50% regression, which was sustained on follow-up imaging. Five patients underwent LT, and at explant pathology, tumor necrosis ranging between 40% and 90% was demonstrated. No patients showed tumor recurrence after LT (median follow-up period of 6 mo). The main conclusions of the paper were that CRT is a safe and efficacious local bridging therapy for patients with HCC who are on the waiting list for LT and that further studies are warranted to compare the effectiveness of CRT to other local treatment regimens[91].

Combined treatments

Experiences with combined therapies such as TACE followed by RFA[92-94] or RFA shortly after TACE[95] have been published in recent years, typically in the setting of unresectable HCC larger than 3 cm. The rationale for the use of combined treatment rather than a single treatment is to reach a higher local tumor control rate due to higher rates of complete tumor necrosis. In this context, the question arises of how TACE and RFA should be sequenced. The advantage of performing TACE prior to RFA is the reduced heat-sink effect with the ability to create larger ablation zones more easily. The advan-

tage of using TACE after RFA is that RFA generates a hyperemic rim surrounding the ablation area, which can consequently be targeted by transarterial means more effectively. The approach of combined treatment may be applied even as a bridge to LT. A recent experience with combined TACE followed by RFA in a series of 44 HCC patients within the MC reported the absence of major complications and a 76.9% rate of complete necrosis in the 16 patients with 26 nodules who underwent LT[96].

IMPACT OF BRIDGING TREATMENTS ON DROPOUT FROM THE LIVER TRANSPLANTATION WAITING LIST

The impact of bridging treatments on waiting list dropout is uncertain due to the absence of prospective comparative studies, but the dropout data of treated patients should be compared with the features of HCC patients awaiting LT without any bridging treatment. In the latter case, the dropout rates were greater than 30% 12 mo after being added to the list[2,3]. In 2006, Lesurtel *et al*[97] published an interesting paper dealing with the usefulness of TACE in HCC patients undergoing LT according to the criteria of evidence-based medicine. The question was whether TACE impacted the waiting list dropout rate. They found insufficient evidence to answer this question. Hayashi *et al*[61] reported a discouraging 35% dropout rate in patients with TNM stage 1 or 2 HCC and a mean waiting time of 340 d after treatment with TACE. Similarly, among 54 listed HCC patients who underwent TACE prior to LT, Maddala *et al*[98] revealed drop-out rates of 15% and 25% at 6 and 12 mo, respectively. However, the most recent series including patients treated with TACE before LT have indicated that the dropout rate due to tumor progression is lower and ranges between 3.0% and 9.3%, with a mean waiting time on the transplantation list exceeding 6 mo in the largest available studies[63,64,69] (Table 2).

Less data are available in this setting for patients submitted to RFA. In a preliminary study published in 2002, Fontana *et al*[99] reported a dropout rate of 21% over a mean waiting period of 7.9 mo among 33 patients treated with RFA prior to LT. In more recent papers including larger numbers of patients, the dropout rate due to HCC progression was found to be 0% after a mean waiting time of 9.5 mo in one study[73] and 5.8% at 12 mo in another study[74] (Table 2). In a large study including only HCC patients within the MC awaiting LT, 77 patients who underwent RFA were compared to 93 patients without any bridging treatment; a non-specific trend toward a higher dropout rate for tumor-specific events was detected among RFA patients (21% *vs* 11%), but the mean waiting time was significantly higher in the RFA group. Using survival analysis modeling, there was no significant difference in the time to dropout between the RFA and no-treatment groups for all causes[78].

Encouraging data have been reported following the application of multimodal schedules of treatment; in a series of 44 listed HCC patients within the MC who systematically underwent TACE followed by RFA, the intention-to-treat cumulative dropout rates were 5.5% and 11.0% at 12 and 24 mo, respectively[96].

Lastly, the short-term response to bridging treatment has recently been reported to be crucial in the prediction of dropout. In a recent report by De Giorgio *et al*[24], 170 HCC patients awaiting LT within the MC who underwent percutaneous ablation, TACE, or surgery as a bridging treatment were analyzed. Total tumor diameter and recurrence or persistence of tumor activity at the 6-wk follow-up after therapy were significantly correlated with progression beyond the MC and dropout from the waiting list. The finding of a significantly decreased dropout probability among T2 patients achieving a complete or partial response to bridging treatment compared with patients with an inadequate or no response to treatment has also been confirmed in two other large studies[10,14].

In summary, there is sufficient evidence to conclude that bridging treatments yielding complete or subtotal HCC necrosis on imaging effectively reduce the rate of dropout from the waiting list.

IMPACT OF BRIDGING TREATMENTS ON RECURRENCE OF HEPATOCELLULAR CARCINOMA AFTER LIVER TRANSPLANTATION

A less than 15% recurrence rate has been reported for HCC in patients within the MC undergoing LT without any treatment[30]. Whether the application of bridging therapies while on the waiting list decreases this rate is controversial. Again, prospective multicenter comparative studies are lacking in this field, and the only available data were obtained in single-center retrospective case series.

Regarding TACE, in a cohort of 111 HCC patients undergoing LT (54 treated preoperatively with TACE), Majno *et al*[59] showed that downstaging of tumors > 3 cm and total necrosis of the nodule at explant analysis were associated with better 5-year disease-free survival than either an inadequate response to TACE or no TACE before LT. Thereafter, low recurrence rates of 7.6% and 10.7% were reported in two large series of HCC patients within the MC who were treated with TACE before LT[63,64]. A clear trend toward longer recurrence-free survival has also been observed by Milllonig *et al*[63] in patients with complete tumor necrosis when compared with patients with viable tumor at explant analysis. More recently, Tsochatzis *et al*[67] evaluated 150 consecutive patients with HCC within the MC who underwent LT. Sixty-seven patients (45%) underwent transarterial embolization (TAE) with polyvinyl alcohol particles or TACE before LT, and the remaining 83 patients were not treated before

Table 2 Selected studies on non-surgical bridging therapy for hepatocellular carcinoma before liver transplantation *n* (%)

Ref.	Treatment	Patients	HCC stage	Dropout rate -Total -HCC progression	HCC recurrence after LT	Intention-to-treat survival	Survival after LT
Fontana *et al*[99]	RFA	33 (15 LT)	MC (30 pts)	NA	2 (13)	NA	85% at 3 yr
Graziadei *et al*[60]	TACE	48 (41 LT)	MC	0	1 (2.4)	94% at 5 yr	94% at 5 yr
Hayashi *et al*[61]	TACE	20 (12 LT)	MC	6 (35)	NA	61% at 3 yr	100% at 4 yr
Maddala *et al*[98]	TACE	54 (46 LT)	MC (47 pts)	8 (14.8) 6 (11.1)	5 (13.3)	61% at 5 yr	74% at 5 yr
Mazzaferro *et al*[73]	RFA	50 (50 LT)	MC (40 pts)	0 (0)	2 (4)	NA	83% at 3 yr
Lu *et al*[74]	RFA	52 (41 LT)	MC (42 pts)	6 (12) 3 (5.8)	0 (0)	74% at 3 yr	76% at 3 yr
Castrogaudin *et al*[81]	PEI	34 (23 LT)	UNOS T1-T2 (30 pts)	5 (14.7) 2 (5.9)	1 (4.3)	NA	19/23 (82.6%) alive (median FU 21 mo)
Pompili *et al*[75]	RFA, PEI	40 (40 LT)	MC (37 pts)	NA	3 (7.5)	NA	85.4% at 3 yr
Porrett *et al*[79]	TACE, RFA, TARE	31 (31 LT)	UNOS T1-T2	NA	7 (22.6)	NA	84% at 3 yr
Brillet *et al*[76]	RFA	21 (16 LT)	MC	5 (23.8) 3 (14.3)	1 (6.3)	NA	11/16 (69%) alive (median FU 25 mo)
Millonig *et al*[63]	TACE	68 (66 LT)	MC	2 (3)	5 (7.6)	70% at 5 yr	NA
Majno *et al*[69]	TACE	43 (43 LT)	MC	12 (27.9) 4 (9.3)	4 (9.3)	NA	NA
Rodríguez-Sanjuán *et al*[77]	RFA	28 (28 LT)	MC (25 pts)	NA	2 (7.1)	NA	NA
Alba *et al*[64]	TACE	63 (56 LT)	MC	7 (11) 3 (4.8)	6 (10.7)	NA	60.4% at 5 yr
Branco *et al*[82]	PEI	62 (59 LT)	MC	3 (4.8)	3 (5.1)	64.4% at 3 yr	67.7% at 3 yr
DuBay *et al*[78]	RFA	77 (51 LT)	MC	19 (25) 16 (21)	1 (2)	NA	> 80% at 3 yr
Ashoori *et al*[96]	TACE + RFA	36 (16 LT)	MC	6 (16.7) 4 (11.1)	0 (0)	NA	11/16 alive (median FU 29.9 mo)
Tsochatzis *et al*[67]	TACE, TAE	67 (67 LT)	MC	NA	4 (6)	NA	NA

HCC: Hepatocellular carcinoma; LT: Liver transplantation; RFA: Radiofrequency ablation; MC: Milan criteria; NA: Not available; TACE: Transarterial chemoembolization; PEI: Percutaneous ethanol injection; UNOS: United Network for Organ Sharing; TARE: Transarterial radio embolization; TAE: Transarterial embolization.

LT. HCC recurrence after LT was significantly lower in the TAE-TACE group (6%) than in the no TAE-TACE group (18.1%) (Table 2). Furthermore, post-transplant HCC recurrence was independently associated with no neo-adjuvant transarterial therapy and the total radiological size of the HCC nodules.

HCC recurrence after LT has been evaluated in 7 studies of patients who underwent RFA as the only bridging treatment. In total, 231 patients were evaluated over follow-up periods of 15-41 mo (mean 28 mo). Overall, HCC recurrence was detected in 8 patients (3.5%), and the rate of recurrence ranged between 0% and 13%[73-78,99] (Table 2).

Some recent single-center studies appear to confirm a positive impact of bridging treatments on HCC recurrence after LT. In a series of 147 HCC candidates (38% outside the MC) who underwent RFA, TACE, or multimodal treatment before LT, a complete or partial response was observed in 57.8% of cases. Transplanted patients with stable disease or no response to pre-LT HCC treatment had a significant 6-fold increase in tumor recurrence after LT compared with patients with a complete or partial response (13% *vs* 2%)[10]. In another study that included 315 HCC patients who were candidates for LT (17% outside the MC) and underwent TACE, RFA, PEI, or surgical resection, a complete response to treatment was observed in 49.1% of cases; transplanted patients with a partial or no response to bridging treatments showed a significantly higher risk of HCC recurrence compared with patients with a complete response (19.4% *vs* 5.5%)[14]. Among 137 transplanted patients (42 outside the MC) who underwent locoregional bridging treatments such as resection, TACE, RFA, and PEI before LT, AFP > 400 ng/mL was the only significant pre-transplant factor linked to HCC recurrence after LT. Conversely, the use of locoregional treatments was a significant protective factor, and the best 5-year tumor-free survival was observed in patients within the MC who underwent locoregional treatment[100]. Lastly, within a group of 93 consecutive HCC patients (36 beyond MC) who underwent LT, 59 underwent pre-transplant TACE or RFA. The 5-year tumor-free survival did not significantly differ between treated and untreated patients (78% *vs* 68%). However, among the treated patients, the presence of more than 50% necrosis of the target lesions at explant analysis was associated with a significantly better 5-year tumor-free survival rate (96% *vs* 21%)[101].

Overall, a trend toward a decreased recurrence rate after LT appears to emerge in patients achieving a total or subtotal response to the treatment administered before LT.

IMPACT OF BRIDGING TREATMENTS ON SURVIVAL AFTER LIVER TRANSPLANTATION

Independent of the treatment administered, a key question remains to be answered: do bridging treatments improve survival in HCC patients who undergo LT? There is insufficient evidence of a beneficial effect of TACE because data obtained from prospective randomized studies are lacking[97]. A multicenter retrospective case control study from France compared 100 HCC patients who underwent TACE before transplantation and 100 HCC patients transplanted without any prior treatment. The 5-year survival (59% in both groups) and 5-year disease free survival (69% *vs* 64%) rates were not significantly different. At explant analysis, greater than 80% total or subtotal HCC necrosis was found in 30% of treated patients, and this subgroup showed a non-significant trend toward a better 5-year survival compared with a matched untreated control group (63% *vs* 54%)[62]. It can be reasonably argued that patients with total/subtotal tumor necrosis might receive a significant survival benefit from TACE before LT, perhaps due to a decreased risk of post-transplant HCC recurrence. This hypothesis appears to have been confirmed in a study by Millonig *et al*[63] that included 116 HCC patients who underwent TACE before transplantation. Most of the patients were within the MC, and complete tumor necrosis was found in 27% of the cases. The 5-year survival rate was higher in patients with completely necrotic tumors than in patients with partial necrosis (86% *vs* 66%), although this difference did not reach statistical significance.

The influence of neo-adjuvant treatments on post-LT survival should be analyzed independent of the treatment used; however, the only available data come from single-center retrospective series and provide contradictory results. In a study by Bharat *et al*[102], 46 HCC patients undergoing various bridging treatments before LT were compared to 46 matched HCC patients transplanted without any treatment. The 5-year survival rate was significantly higher in the treated group (82% *vs* 52%), although the survival advantage was evident only for patients with T2-T4 tumors, not for patients with T0-T1 tumors. Even the 5-year disease-free survival rate was slightly higher in the treated group (84% *vs* 76%), although this difference was not significant. In a study by Lao *et al*[103], 91 untreated HCC patients who underwent LT were compared to 33 patients with HCC who underwent TACE, RFA, or PEI before LT. HCC recurred only in 9 untreated patients, and the only factors significantly linked to tumor recurrence were a MELD score < 14, AFP > 1000 ng/mL, and the absence of pre-LT bridging treatment. The disease-free survival showed a non-significant trend toward a better outcome in treated patients, whereas the cumulative survival did not differ. Heckman *et al*[104] compared the outcomes of 50 HCC patients undergoing bridging therapy before LT to those of 73 HCC patients transplanted without any prior treatment; they found a non-significant trend toward improved 5-year survival in treated patients (81% *vs* 71%). Porrett *et al*[79] compared 30 treated patients to 33 untreated patients before transplant. Their study failed to show any survival difference between the groups, but it should be noted that only 20% of the treated patients had complete HCC necrosis at explant analysis. Lastly, in the previously cited study by DuBay *et al*[78], no differences in 5-year overall or tumor-free survival from the list date or transplant were identified when comparing 77 patients treated with RFA to 93 matched untreated patients. No data were provided about the achievement of complete necrosis in the ablated tumors at explant analysis.

Although a definitive conclusion cannot be made, a positive impact of pre-LT treatments on post-LT survival could be present. Indeed, in the United States, data on liver transplant activity for HCC from 1997 to 2006 demonstrated a higher 3-year post-LT survival in patients who underwent ablative procedures compared with patients who did not[105]. Moreover, studies reporting no difference between treated and untreated patients also tend to report shorter waiting times for LT[29].

DOWNSTAGING OF HCC BEYOND THE CONVENTIONAL LIVER TRANSPLANTATION CRITERIA

Downstaging of HCC to within the MC or the UCSF criteria is an attractive alternative to expanding the tumor size limits for LT. Theoretically, the downstaging process allows the selection of tumors with a more favorable biology that will likely respond to downstaging treatments and will also do well following LT[106].

In recent years, several papers have been published defining successful downstaging as fulfilling the MC[107-114] or the UCSF criteria[106]. However, different criteria for successful treatment have been used in other studies, including fulfilling the MC without a serum AFP level higher than 400 ng/mL[115], a 30%-50% decrease in the size of treated nodules[60,116], or no tumor progression during the downstaging treatment in patients with well or moderately differentiated HCC[4,117] (Table 3). In some of these studies, only TACE[60,108,110,112-114] or transarterial chemoinfusion[107] was used as the downstaging procedure, whereas in other studies, a multimodal approach was used, including TACE, RFA, PEI, or surgical resection[4,106,114,115,117]. TARE as a single downstaging procedure was retrospectively compared to TACE in a study by Lewandowski *et al*[109]. Better performance was observed for TARE in terms of the downstaging success rate and 3-year intention-to-treat post-HCC treatment survival.

Significant factors for unsuccessful downstaging related to biological tumor features have been reported by some of these papers. In the study by Yao *et al*[106], AFP > 1000 ng/mL was the only significant negative prognostic factor. Barakat *et al*[111] showed that the mean

Table 3 Selected studies on downstaging therapy for hepatocellular carcinoma before liver transplantation *n* (%)

Ref.	Treatment	Pts	Inclusion criteria[1]	Successful downstage -Criteria -Rate	Transplanted pts	Recurrence free survival after LT	Intention to treat survival	Survival after LT
Graziadei *et al*[60]	TACE	36	HCC > 5 cm	Decreased size > 50% 11/36 (31)	10	Recurrent HCC: 3 pts (30)	31% at 5 yr	41% at 4 yr
Otto *et al*[116]	TACE	62	Beyond MC	Decreased size ≥ 30% 34/62 (55)	27	68% at 5 yr	NA	73.2% at 5 yr
Cillo *et al*[4]	TACE, RFA, PEI, Resection	40	Beyond MC WD or MD HCC	Maintenance of selection criteria NA	31	Recurrent HCC: 0 pts	79% at 5 yr	> 90% at 3 yr
Chapman *et al*[108]	TACE	76	Beyond MC	MC 18/76 (24)	17	50% at 5 yr	NA	93.8% at 5 yr
Yao *et al*[106]	TACE, RFA, Resection	61	1 HCC 5-8 cm 2-3 HCCs 3-5 cm, total diameter ≤ 8 cm 4-5 HCCs ≤ 3 cm total diameter ≤ 8 cm	UCSF 43/61 (71)	35	92% at 2 yr	69% at 4 yr	92% at 2 yr
Ravaioli *et al*[115]	Multimodal (TACE, PEI, RFA, Resection)	48	1 HCC 5-8 cm 2 HCCs 3-5 cm, total diameter ≤ 8 cm 3-5 HCCs ≤ 4 cm total diameter ≤ 12 cm	MC and AFP < 400 ng/mL 32/48 (67)	32	71% at 3 yr	62% at 3 yr	NA
Lewandowski *et al*[109]	TACE (43 patients) TARE (43 patients)	86	UNOS T3	MC TACE 11/35 (31) TARE 25/43 (58)	TACE 11 TARE 9	TACE 73% at 1 yr TARE 89% at 1 yr	TACE 19% at 3 yr TARE 59% at 3 yr	NA
De Luna *et al*[107]	TACI	27	Beyond MC	MC 17/27 (63)	15	NA	84.1% at 3 yr	78.8% at 3 yr
Jang *et al*[110]	TACE	386	Beyond MC	MC or complete tumor necrosis 160/386 (41.5)	37	66.3% at 5 yr	NA	54.6% at 5 yr
Barakat *et al*[111]	TACE, TARE, RFA, Resection	32	Beyond UCSF (18 pts) Beyond MC (14 pts)	UNOS T2 18/32 (56.3)	13	Recurrent HCC: 2 pts (15.4%)	NA	75% at 2 yr
Bargellini *et al*[112]	TACE	33	Beyond MC	Complete or partial response, or stable disease according to mRECIST criteria NA	33	74.4% at 5 yr	NA	72.5% at 5 yr
Bova *et al*[113]	TACE, TAE	48	Beyond MC	MC AFP < 100 ng/mL 19/48 (39)	9	Recurrent HCC: 1 pt (11.1%)	NA	NA
Lei *et al*[114]	TACE, RFA, Resection, HIFU	58	Beyond MC Within UCSF	MC NA	58	63.8% at 5 yr	NA	74.1% at 5 yr

[1]Patients with vascular invasion or extrahepatic tumor spread at baseline excluded in all series. HCC: Hepatocellular carcinoma; LT: Liver transplantation; TACE: Transarterial chemoembolization; MC: Milan criteria; NA: Not available; RFA: Radiofrequency ablation; PEI: Percutaneous ethanol injection; WD: Well differentiated; MD: Moderately differentiated; UCSF: University of California San Francisco; AFP: α-fetoprotein; TARE: Transarterial radio embolization; UNOS: United Network for Organ Sharing; TACI: Transarterial chemoinfusion; mRECIST: Modified Response Evaluation Criteria in Solid Tumors; TAE: Transarterial embolization; HIFU: High-intensity focused ultrasound; pts: Patients.

AFP level and the rate of infiltrative tumors were significantly higher in patients who did not achieve successful downstaging. An AFP level lower than 100 ng/mL and the 3-year survival probability calculated using the Metroticket calculator[33] were the only independent predictors of successful downstaging in the study by Bova *et al*[113]. An AFP slope > 15 ng/mL per month and tumor progression according to the Modified Response Evaluation Criteria in Solid Tumors (mRECIST)[118] were independent risk factors for HCC recurrence and patient death in an international retrospective multicenter European study performed by Lai *et al*[25] that included MC-within (316 cases) and MC-outside (116 cases) patients who underwent LT after locoregional therapy. We should also highlight that after successful downstaging, some authors have recommended that patients undergo a 3-mo observation period before listing to assess the stability of neoplastic disease[4,106,115]. This "test of time" will identify rapidly recurring lesions, vascular invasion, and distant metastasis, thereby decreasing the risk of tumor recurrence and poor overall results after LT[34].

Overall, according to the presently available data, the successful downstaging rate ranges between 24% and 71% (Table 3). The proportion of patients transplanted

ranges between 10% and 67%, and the average waiting time to LT ranges between 2 and 10.9 mo[29]. Additionally, the reported survival rates range from 78.8% to more than 90% and from 54.6% to 93.8% at 3 and 5 years, respectively[119]. Two prospective studies have demonstrated that survival after LT in patients with large tumors successfully downstaged within the MC[115] or the UCSF criteria[106] is similar to that of patients who initially met the criteria for transplantation. Six studies[4,60,107,108,114,116] compared patients who were downstaged successfully within the MC with those who initially met the MC. Five of these studies[4,107,108,114,116] reported no significant difference in absolute or disease-free survival between groups, whereas one study[60] reported that patients who were downstaged successfully had significantly worse survival at 1, 2, and 5 years after LT. Lastly, in a recent study, no significant differences in postoperative complications, tumor recurrence, or survival rate were reported between two groups of patients with advanced HCC who underwent deceased donor LT (52 patients) or living donor LT (31 patients) after successful downstaging therapy[42].

CONCLUSION

Currently, locoregional therapies play a crucial role in the treatment of patients awaiting LT. For patients listed within the MC (stage T2-HCC), a delay of LT over 6-12 mo without bridging treatment is a well-recognized risk factor for tumor progression and dropout from the list or interval dissemination with post-transplant tumor recurrence[2,3,16]. For this reason, the optimal strategy for T2-HCC patients awaiting LT should be to transplant within 6 mo without pre-transplant therapy[120]. However, if a longer waiting time is needed, following the current guidelines of the American Association for the Study of Liver Disease and the European Association for the Study of the Liver for the treatment of HCC[121,122] and the recommendations of a recent international consensus conference on the management of HCC patients who are LT candidates[123], the use of bridging treatments is recommended, as several studies in recent years have documented their usefulness in preventing tumor progression. There is, however, no evidence that bridging treatments are useful in patients with T1-HCC[123].

In patients who underwent previous liver resection and experienced tumor recurrence but are within the currently accepted transplant criteria or those with liver function failure, salvage LT using deceased donor livers yields an acceptable long-term survival rate and can be considered[6,25]. Salvage LT using living donors has also been successfully performed in centers with high-volume living donor programs, and they appear to provide long-term results comparable to those obtained using deceased donor grafts[48].

Regarding non-surgical bridging therapies, no recommendation can be made for one type of locoregional therapy over others[123]. However, RFA could be the first-line treatment for lesions up to 3 cm, in which complete tumor necrosis has been shown in more than 50% of cases at explant analysis. The risk of major complications related to RFA in this patient setting appears to be quite low, but it is good clinical practice to limit needle insertions and to avoid the treatment of superficially located lesions. PEI appears to show lower efficacy and can be reserved for small lesions located in sites considered "dangerous" for RFA (*e.g.*, near the gallbladder or bowel loops). TACE should be preferred for treating lesions > 3 cm because its effectiveness appears to be better in well-vascularized tumors with large feeding arteries; selective and superselective TACE should be preferred, and the possible advantage of DEBs-TACE over lipiodol-TACE should be investigated in future studies. Multimodal treatment strategies, including sequentially applied TACE and RFA, appear to be promising, although the role of alternative treatments such as PLA, MWA, TARE, and CRT needs to be investigated in a larger number of patients. Regardless, all ablation procedures should be better evaluated with caution in patients with decompensated liver function to avoid irreversible liver failure and severe complications precluding LT.

The response of HCC to neoadjuvant treatments should be evaluated using the mRECIST criteria[118]. The RECIST criteria[124] were amended to the mRECIST in 2008[125] in the setting of HCC based on the concept that the evaluation of the treatment response should consider the amount of necrosis when estimating the decreased tumor load, not only the reduction in tumor size. However, it should also be considered that computed tomography and magnetic resonance imaging, which are currently used to assess the results of the ablation bridging procedures, tend to overestimate treatment effectiveness. In several studies, the concordance in the diagnosis of complete necrosis between the last imaging evaluation before LT and the pathological assessment at explant analysis of the target lesions has been reported to range between 50% and 83%; this is primarily due to the persistence of microscopic avascular neoplastic foci that are primarily located peripherally and cannot be detected by contrast-enhanced imaging techniques[74,75,77,85,106,115,126,127].

Although no solid conclusions may be drawn due to the absence of prospective comparative studies, it appears reasonable to state that bridging treatments decrease the dropout rate from the waiting list of T2-HCC patients and could have a positive impact on post-LT HCC recurrence and overall survival, at least in patients with complete or subtotal necrosis of the targeted lesions and a longer waiting period[29,105]. Furthermore, the response to pre-LT treatments may represent a surrogate marker of tumor biology and should be considered in the selection and prioritization of candidates for LT. That is, the transplant priority of T2-HCC candidates could be reduced after successful bridging therapy and a 3-6 mo period of observation confirming inactive neoplastic disease, and patients showing stable or progressive disease after treatment could then be prioritized.

However, if an advantage is given on the waiting list to non-responding patients, a worsening in the outcome of LT in terms of overall survival, primarily due to an increased incidence of HCC recurrence after transplantation, should be considered. Whether this risk is acceptable is a matter of debate, and this issue should be further addressed in future studies[14,29,128].

HCC downstaging using exclusively TACE or multimodal sequential therapies to meet the conventional criteria for LT among carefully selected patients yields promising results in terms of overall and disease-free survival. In particular, some recent papers have demonstrated that patients successfully downstaged within the MC or the UCSF criteria can achieve a 5-year survival rate comparable to that of patients meeting the above-mentioned criteria without requiring downstaging[123]. A follow-up period of 3 mo demonstrating stable disease after successful downstaging is suggested before inclusion on the waiting list for transplantation.

REFERENCES

1 **Mazzaferro V**, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, Montalto F, Ammatuna M, Morabito A, Gennari L. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med* 1996; **334**: 693-699 [PMID: 8594428 DOI: 10.1056/NEJM199603143341104]

2 **Llovet JM**, Fuster J, Bruix J. Intention-to-treat analysis of surgical treatment for early hepatocellular carcinoma: resection versus transplantation. *Hepatology* 1999; **30**: 1434-1440 [PMID: 10573522 DOI: 10.1002/hep.510300629]

3 **Yao FY**, Bass NM, Nikolai B, Davern TJ, Kerlan R, Wu V, Ascher NL, Roberts JP. Liver transplantation for hepatocellular carcinoma: analysis of survival according to the intention-to-treat principle and dropout from the waiting list. *Liver Transpl* 2002; **8**: 873-883 [PMID: 12360427 DOI: 10.1053/jlts.2002.34923]

4 **Cillo U**, Vitale A, Grigoletto F, Gringeri E, D'Amico F, Valmasoni M, Brolese A, Zanus G, Srsen N, Carraro A, Burra P, Farinati F, Angeli P, D'Amico DF. Intention-to-treat analysis of liver transplantation in selected, aggressively treated HCC patients exceeding the Milan criteria. *Am J Transplant* 2007; **7**: 972-981 [PMID: 17391137 DOI: 10.1111/j.1600-6143.2006.01719.x]

5 **Kadry Z**, Schaefer EW, Uemura T, Shah AR, Schreibman I, Riley TR. Impact of geographic disparity on liver allocation for hepatocellular cancer in the United States. *J Hepatol* 2012; **56**: 618-625 [PMID: 22027581 DOI: 10.1016/j.jhep.2011.08.019]

6 **Lai Q**, Avolio AW, Lerut J, Singh G, Chan SC, Berloco PB, Tisone G, Agnes S, Chok KS, Sharr W, Rossi M, Manzia TM, Lo CM. Recurrence of hepatocellular cancer after liver transplantation: the role of primary resection and salvage transplantation in East and West. *J Hepatol* 2012; **57**: 974-979 [PMID: 22771712 DOI: 10.1016/j.jhep.2012.06.033]

7 **Toso C**, Dupuis-Lozeron E, Majno P, Berney T, Kneteman NM, Perneger T, Morel P, Mentha G, Combescure C. A model for dropout assessment of candidates with or without hepatocellular carcinoma on a common liver transplant waiting list. *Hepatology* 2012; **56**: 149-156 [PMID: 22271250 DOI: 10.1002/hep.25603]

8 **Vitale A**, Morales RR, Zanus G, Farinati F, Burra P, Angeli P, Frigo AC, Del Poggio P, Rapaccini G, Di Nolfo MA, Benvegnù L, Zoli M, Borzio F, Giannini EG, Caturelli E, Chiaramonte M, Trevisani F, Cillo U. Barcelona Clinic Liver Cancer staging and transplant survival benefit for patients with hepatocellular carcinoma: a multicentre, cohort study. *Lancet Oncol* 2011; **12**: 654-662 [PMID: 21684210 DOI: 10.1016/S1470-2045(11)70144-9]

9 **Avolio AW**, Cillo U, Salizzoni M, De Carlis L, Colledan M, Gerunda GE, Mazzaferro V, Tisone G, Romagnoli R, Caccamo L, Rossi M, Vitale A, Cucchetti A, Lupo L, Gruttadauria S, Nicolotti N, Burra P, Gasbarrini A, Agnes S. Balancing donor and recipient risk factors in liver transplantation: the value of D-MELD with particular reference to HCV recipients. *Am J Transplant* 2011; **11**: 2724-2736 [PMID: 21920017 DOI: 10.1111/j.1600-6143.2011.03732.x]

10 **Vitale A**, D'Amico F, Frigo AC, Grigoletto F, Brolese A, Zanus G, Neri D, Carraro A, D'Amico FE, Burra P, Russo F, Angeli P, Cillo U. Response to therapy as a criterion for awarding priority to patients with hepatocellular carcinoma awaiting liver transplantation. *Ann Surg Oncol* 2010; **17**: 2290-2302 [PMID: 20217249 DOI: 10.1245/s10434-010-0993-4]

11 **Avolio AW**, Siciliano M, Barbarino R, Nure E, Annicchiarico BE, Gasbarrini A, Agnes S, Castagneto M. Donor risk index and organ patient index as predictors of graft survival after liver transplantation. *Transplant Proc* 2008; **40**: 1899-1902 [PMID: 18675083 DOI: 10.1016/j.transproceed.2008.05.070]

12 **Wiesner RH**, Freeman RB, Mulligan DC. Liver transplantation for hepatocellular cancer: the impact of the MELD allocation policy. *Gastroenterology* 2004; **127**: S261-S267 [PMID: 15508092 DOI: 10.1053/j.gastro.2004.09.040]

13 **Roayaie K**, Feng S. Allocation policy for hepatocellular carcinoma in the MELD era: room for improvement? *Liver Transpl* 2007; **13**: S36-S43 [PMID: 17969067 DOI: 10.1002/lt.21329]

14 **Cucchetti A**, Cescon M, Bigonzi E, Piscaglia F, Golfieri R, Ercolani G, Cristina Morelli M, Ravaioli M, Daniele Pinna A. Priority of candidates with hepatocellular carcinoma awaiting liver transplantation can be reduced after successful bridge therapy. *Liver Transpl* 2011; **17**: 1344-1354 [PMID: 21837731 DOI: 10.1002/lt.22397]

15 **Del Gaudio M**, Ercolani G, Ravaioli M, Cescon M, Lauro A, Vivarelli M, Zanello M, Cucchetti A, Vetrone G, Tuci F, Ramacciato G, Grazi GL, Pinna AD. Liver transplantation for recurrent hepatocellular carcinoma on cirrhosis after liver resection: University of Bologna experience. *Am J Transplant* 2008; **8**: 1177-1185 [PMID: 18444925 DOI: 10.1111/j.1600-6143.2008.02229.x]

16 **Freeman RB**, Edwards EB, Harper AM. Waiting list removal rates among patients with chronic and malignant liver diseases. *Am J Transplant* 2006; **6**: 1416-1421 [PMID: 16686765 DOI: 10.1111/j.1600-6143.2006.01321.x]

17 **Washburn K**, Edwards E, Harper A, Freeman R. Hepatocellular carcinoma patients are advantaged in the current liver transplant allocation system. *Am J Transplant* 2010; **10**: 1643-1648 [PMID: 20486906 DOI: 10.1111/j.1600-6143.2010.03127.x]

18 **Pomfret EA**, Washburn K, Wald C, Nalesnik MA, Douglas D, Russo M, Roberts J, Reich DJ, Schwartz ME, Mieles L, Lee FT, Florman S, Yao F, Harper A, Edwards E, Freeman R, Lake J. Report of a national conference on liver allocation in patients with hepatocellular carcinoma in the United States. *Liver Transpl* 2010; **16**: 262-278 [PMID: 20209641 DOI: 10.1002/lt.21999]

19 **Cillo U**, Vitale A, Volk ML, Frigo AC, Grigoletto F, Brolese A, Zanus G, D'Amico F, Farinati F, Burra P, Russo F, Angeli P, D'Amico DF. The survival benefit of liver transplantation in hepatocellular carcinoma patients. *Dig Liver Dis* 2010; **42**: 642-649 [PMID: 20381438 DOI: 10.1016/j.dld.2010.02.010]

20 **Ravaioli M**, Grazi GL, Dazzi A, Bertuzzo V, Ercolani G, Cescon M, Cucchetti A, Masetti M, Ramacciato G, Pinna AD. Survival benefit after liver transplantation: a single European center experience. *Transplantation* 2009; **88**: 826-834 [PMID: 19920783 DOI: 10.1097/TP.0b013e3181b26807]

21 **Avolio AW**, Siciliano M, Barone M, Lai Q, Caracciolo GL, Barbarino R, Nicolotti N, Lirosi MC, Gasbarrini A, Agnes S.

Model for end-stage liver disease dynamic stratification of survival benefit. *Transplant Proc* 2012; **44**: 1851-1856 [PMID: 22974854 DOI: 10.1016/j.transproceed.2012.06.056]
22 **Merani S**, Majno P, Kneteman NM, Berney T, Morel P, Mentha G, Toso C. The impact of waiting list α-fetoprotein changes on the outcome of liver transplant for hepatocellular carcinoma. *J Hepatol* 2011; **55**: 814-819 [PMID: 21334400 DOI: 10.1016/j.jhep.2010.12.040]
23 **Mailey B**, Artinyan A, Khalili J, Denitz J, Sanchez-Luege N, Sun CL, Bhatia S, Nissen N, Colquhoun SD, Kim J. Evaluation of absolute serum α-fetoprotein levels in liver transplant for hepatocellular cancer. *Arch Surg* 2011; **146**: 26-33 [PMID: 21242442 DOI: 10.1001/archsurg.2010.295]
24 **De Giorgio M**, Vezzoli S, Cohen E, Armellini E, Lucà MG, Verga G, Pinelli D, Nani R, Valsecchi MG, Antolini L, Colledan M, Fagiuoli S, Strazzabosco M. Prediction of progression-free survival in patients presenting with hepatocellular carcinoma within the Milan criteria. *Liver Transpl* 2010; **16**: 503-512 [PMID: 20373461 DOI: 10.1002/lt.22039]
25 **Lai Q**, Avolio AW, Graziadei I, Otto G, Rossi M, Tisone G, Goffette P, Vogel W, Pitton MB, Lerut J. α-fetoprotein and modified response evaluation criteria in Solid Tumors progression after locoregional therapy as predictors of hepatocellular cancer recurrence and death after transplantation. *Liver Transpl* 2013; **19**: 1108-1118 [PMID: 23873764 DOI: 10.1002/lt.23706]
26 **Knight M**, Barber K, Gimson A, Collett D, Neuberger J. Implications of changing the minimal survival benefit in liver transplantation. *Liver Transpl* 2012; **18**: 549-557 [PMID: 22238251 DOI: 10.1002/lt.23380]
27 **Merion RM**, Schaubel DE, Dykstra DM, Freeman RB, Port FK, Wolfe RA. The survival benefit of liver transplantation. *Am J Transplant* 2005; **5**: 307-313 [PMID: 15643990 DOI: 10.1111/j.1600-6143.2004.00703.x]
28 **Schaubel DE**, Sima CS, Goodrich NP, Feng S, Merion RM. The survival benefit of deceased donor liver transplantation as a function of candidate disease severity and donor quality. *Am J Transplant* 2008; **8**: 419-425 [PMID: 18190658 DOI: 10.1111/j.1600-6143.2007.02086.x]
29 **Cescon M**, Cucchetti A, Ravaioli M, Pinna AD. Hepatocellular carcinoma locoregional therapies for patients in the waiting list. Impact on transplantability and recurrence rate. *J Hepatol* 2013; **58**: 609-618 [PMID: 23041304 DOI: 10.1016/j.jhep.2012.09.021]
30 **Llovet JM**, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003; **362**: 1907-1917 [PMID: 14667750 DOI: 10.1016/S0140-6736(03)14964-1]
31 **Silva MF**, Sherman M. Criteria for liver transplantation for HCC: what should the limits be? *J Hepatol* 2011; **55**: 1137-1147 [PMID: 21718672 DOI: 10.1016/j.jhep.2011.05.012]
32 **Yao FY**, Ferrell L, Bass NM, Watson JJ, Bacchetti P, Venook A, Ascher NL, Roberts JP. Liver transplantation for hepatocellular carcinoma: expansion of the tumor size limits does not adversely impact survival. *Hepatology* 2001; **33**: 1394-1403 [PMID: 11391528 DOI: 10.1053/jhep.2001.24563]
33 **Mazzaferro V**, Llovet JM, Miceli R, Bhoori S, Schiavo M, Mariani L, Camerini T, Roayaie S, Schwartz ME, Grazi GL, Adam R, Neuhaus P, Salizzoni M, Bruix J, Forner A, De Carlis L, Cillo U, Burroughs AK, Troisi R, Rossi M, Gerunda GE, Lerut J, Belghiti J, Boin I, Gugenheim J, Rochling F, Van Hoek B, Majno P. Predicting survival after liver transplantation in patients with hepatocellular carcinoma beyond the Milan criteria: a retrospective, exploratory analysis. *Lancet Oncol* 2009; **10**: 35-43 [PMID: 19058754 DOI: 10.1016/S1470-2045(08)70284-5]
34 **Toso C**, Mentha G, Kneteman NM, Majno P. The place of downstaging for hepatocellular carcinoma. *J Hepatol* 2010; **52**: 930-936 [PMID: 20385428 DOI: 10.1016/j.jhep.2009.12.032]
35 **Sala M**, Fuster J, Llovet JM, Navasa M, Solé M, Varela M, Pons F, Rimola A, García-Valdecasas JC, Brú C, Bruix J. High pathological risk of recurrence after surgical resection for hepatocellular carcinoma: an indication for salvage liver transplantation. *Liver Transpl* 2004; **10**: 1294-1300 [PMID: 15376311 DOI: 10.1002/lt.20202]
36 **Earl TM**, Chapman WC. Hepatocellular Carcinoma: resection versus transplantation. *Semin Liver Dis* 2013; **33**: 282-292 [PMID: 23943108 DOI: 10.1055/s-0033-1351783]
37 **Lei JY**, Yan LN, Wang WT. Transplantation vs resection for hepatocellular carcinoma with compensated liver function after downstaging therapy. *World J Gastroenterol* 2013; **19**: 4400-4408 [PMID: 23885153 DOI: 10.3748/wjg.v19.i27.4400]
38 **Lee KK**, Kim DG, Moon IS, Lee MD, Park JH. Liver transplantation versus liver resection for the treatment of hepatocellular carcinoma. *J Surg Oncol* 2010; **101**: 47-53 [PMID: 19798686 DOI: 10.1002/jso.21415]
39 **Cillo U**, Vitale A, Dupuis D, Corso S, Neri D, D'Amico F, Gringeri E, Farinati F, Vincenzi V, Zanus G. Laparoscopic ablation of hepatocellular carcinoma in cirrhotic patients unsuitable for liver resection or percutaneous treatment: a cohort study. *PLoS One* 2013; **8**: e57249 [PMID: 23437351]
40 **Majno PE**, Sarasin FP, Mentha G, Hadengue A. Primary liver resection and salvage transplantation or primary liver transplantation in patients with single, small hepatocellular carcinoma and preserved liver function: an outcome-oriented decision analysis. *Hepatology* 2000; **31**: 899-906 [PMID: 10733546]
41 **Barone M**, Avolio AW, Di Leo A, Burra P, Francavilla A. ABO blood group-related waiting list disparities in liver transplant candidates: effect of the MELD adoption. *Transplantation* 2008; **85**: 844-849 [PMID: 18360266 DOI: 10.1097/TP.0b013e318166cc38]
42 **Lei J**, Yan L, Wang W. Comparison of the outcomes of patients who underwent deceased-donor or living-donor liver transplantation after successful downstaging therapy. *Eur J Gastroenterol Hepatol* 2013; **25**: 1340-1346 [PMID: 23652915 DOI: 10.1097/MEG.0b013e3283622743]
43 **Grant RC**, Sandhu L, Dixon PR, Greig PD, Grant DR, McGilvray ID. Living vs. deceased donor liver transplantation for hepatocellular carcinoma: a systematic review and meta-analysis. *Clin Transplant* 2013; **27**: 140-147 [PMID: 23157398 DOI: 10.1111/ctr.12031]
44 **Avolio AW**, Halldorson JB, Burra P, Dutkowski P, Agnes S, Clavien PA. Balancing utility and need by means of donor-to-recipient matching: a challenging problem. *Am J Transplant* 2013; **13**: 522-523 [PMID: 23282243 DOI: 10.1111/ajt.12031]
45 **Adam R**, Azoulay D, Castaing D, Eshkenazy R, Pascal G, Hashizume K, Samuel D, Bismuth H. Liver resection as a bridge to transplantation for hepatocellular carcinoma on cirrhosis: a reasonable strategy? *Ann Surg* 2003; **238**: 508-518; discussion 518-519 [PMID: 14530722 DOI: 10.1097/01.sla.0000090449.87109.44]
46 **Belghiti J**, Cortes A, Abdalla EK, Régimbeau JM, Prakash K, Durand F, Sommacale D, Dondero F, Lesurtel M, Sauvanet A, Farges O, Kianmanesh R. Resection prior to liver transplantation for hepatocellular carcinoma. *Ann Surg* 2003; **238**: 885-892; discussion 892-893 [PMID: 14631225 DOI: 10.1097/01.sla.0000098621.74851.65]
47 **Facciuto ME**, Koneru B, Rocca JP, Wolf DC, Kim-Schluger L, Visintainer P, Klein KM, Chun H, Marvin M, Rozenblit G, Rodriguez-Davalos M, Sheiner PA. Surgical treatment of hepatocellular carcinoma beyond Milan criteria. Results of liver resection, salvage transplantation, and primary liver transplantation. *Ann Surg Oncol* 2008; **15**: 1383-1391 [PMID: 18320284 DOI: 10.1245/s10434-008-9851-z]
48 **Liu F**, Wei Y, Wang W, Chen K, Yan L, Wen T, Zhao J, Xu M, Li B. Salvage liver transplantation for recurrent hepatocellular carcinoma within UCSF criteria after liver resection. *PLoS One* 2012; **7**: e48932 [PMID: 23145027 DOI: 10.1371/journal.pone.0048932]
49 **Abecassis MM**, Fisher RA, Olthoff KM, Freise CE, Rodrigo

DR, Samstein B, Kam I, Merion RM. Complications of living donor hepatic lobectomy--a comprehensive report. *Am J Transplant* 2012; **12**: 1208-1217 [PMID: 22335782 DOI: 10.1111/j.1600-6143.2011.03972.x]
50 **Hwang S**, Lee SG, Moon DB, Ahn CS, Kim KH, Lee YJ, Ha TY, Song GW. Salvage living donor liver transplantation after prior liver resection for hepatocellular carcinoma. *Liver Transpl* 2007; **13**: 741-746 [PMID: 17457860 DOI: 10.1002/lt.21157]
51 **Kaido T**, Mori A, Ogura Y, Hata K, Yoshizawa A, Iida T, Yagi S, Uemoto S. Living donor liver transplantation for recurrent hepatocellular carcinoma after liver resection. *Surgery* 2012; **151**: 55-60 [PMID: 21943635 DOI: 10.1016/j.surg.2011.06.032]
52 **Moon JI**, Kwon CH, Joh JW, Choi GS, Jung GO, Kim JM, Shin M, Choi SJ, Kim SJ, Lee SK. Primary versus salvage living donor liver transplantation for patients with hepatocellular carcinoma: impact of microvascular invasion on survival. *Transplant Proc* 2012; **44**: 487-493 [PMID: 22410053 DOI: 10.1016/j.transproceed.2011.11.009]
53 **Poon RT**, Fan ST, Lo CM, Liu CL, Wong J. Long-term survival and pattern of recurrence after resection of small hepatocellular carcinoma in patients with preserved liver function: implications for a strategy of salvage transplantation. *Ann Surg* 2002; **235**: 373-382 [PMID: 11882759 DOI: 10.1097/00000658-200203000-00009]
54 **Llovet JM**, Brú C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. *Semin Liver Dis* 1999; **19**: 329-338 [PMID: 10518312 DOI: 10.1055/s-2007-1007122]
55 **Llovet JM**, Bruix J. Systematic review of randomized trials for unresectable hepatocellular carcinoma: Chemoembolization improves survival. *Hepatology* 2003; **37**: 429-442 [PMID: 12540794 DOI: 10.1053/jhep.2003.50047]
56 **Marelli L**, Stigliano R, Triantos C, Senzolo M, Cholongitas E, Davies N, Tibballs J, Meyer T, Patch DW, Burroughs AK. Transarterial therapy for hepatocellular carcinoma: which technique is more effective? A systematic review of cohort and randomized studies. *Cardiovasc Intervent Radiol* 2007; **30**: 6-25 [PMID: 17103105 DOI: 10.1007/s00270-006-0062-3]
57 **Varela M**, Real MI, Burrel M, Forner A, Sala M, Brunet M, Ayuso C, Castells L, Montañá X, Llovet JM, Bruix J. Chemoembolization of hepatocellular carcinoma with drug eluting beads: efficacy and doxorubicin pharmacokinetics. *J Hepatol* 2007; **46**: 474-481 [PMID: 17239480 DOI: 10.1016/j.jhep.2006.10.020]
58 **Lammer J**, Malagari K, Vogl T, Pilleul F, Denys A, Watkinson A, Pitton M, Sergent G, Pfammatter T, Terraz S, Benhamou Y, Avajon Y, Gruenberger T, Pomoni M, Langenberger H, Schuchmann M, Dumortier J, Mueller C, Chevallier P, Lencioni R. Prospective randomized study of doxorubicin-eluting-bead embolization in the treatment of hepatocellular carcinoma: results of the PRECISION V study. *Cardiovasc Intervent Radiol* 2010; **33**: 41-52 [PMID: 19908093 DOI: 10.1007/s00270-009-9711-7]
59 **Majno PE**, Adam R, Bismuth H, Castaing D, Ariche A, Krissat J, Perrin H, Azoulay D. Influence of preoperative transarterial lipiodol chemoembolization on resection and transplantation for hepatocellular carcinoma in patients with cirrhosis. *Ann Surg* 1997; **226**: 688-701; discussion 701-703 [PMID: 9409568 DOI: 10.1097/00000658-199712000-00006]
60 **Graziadei IW**, Sandmueller H, Waldenberger P, Koenigsrainer A, Nachbaur K, Jaschke W, Margreiter R, Vogel W. Chemoembolization followed by liver transplantation for hepatocellular carcinoma impedes tumor progression while on the waiting list and leads to excellent outcome. *Liver Transpl* 2003; **9**: 557-563 [PMID: 12783395 DOI: 10.1053/jlts.2003.50106]
61 **Hayashi PH**, Ludkowski M, Forman LM, Osgood M, Johnson S, Kugelmas M, Trotter JF, Bak T, Wachs M, Kam I, Durham J, Everson GT. Hepatic artery chemoembolization for hepatocellular carcinoma in patients listed for liver transplantation. *Am J Transplant* 2004; **4**: 782-787 [PMID: 15084175 DOI: 10.1111/j.1600-6143.2004.00413.x]
62 **Decaens T**, Roudot-Thoraval F, Bresson-Hadni S, Meyer C, Gugenheim J, Durand F, Bernard PH, Boillot O, Boudjema K, Calmus Y, Hardwigsen J, Ducerf C, Pageaux GP, Dharancy S, Chazouilleres O, Dhumeaux D, Cherqui D, Duvoux C. Impact of pretransplantation transarterial chemoembolization on survival and recurrence after liver transplantation for hepatocellular carcinoma. *Liver Transpl* 2005; **11**: 767-775 [PMID: 15973710 DOI: 10.1002/lt.20418]
63 **Millonig G**, Graziadei IW, Freund MC, Jaschke W, Stadlmann S, Ladurner R, Margreiter R, Vogel W. Response to preoperative chemoembolization correlates with outcome after liver transplantation in patients with hepatocellular carcinoma. *Liver Transpl* 2007; **13**: 272-279 [PMID: 17256758 DOI: 10.1002/lt.21033]
64 **Alba E**, Valls C, Dominguez J, Martinez L, Escalante E, Lladó L, Serrano T. Transcatheter arterial chemoembolization in patients with hepatocellular carcinoma on the waiting list for orthotopic liver transplantation. *AJR Am J Roentgenol* 2008; **190**: 1341-1348 [PMID: 18430853 DOI: 10.2214/AJR.07.2972]
65 **Golfieri R**, Cappelli A, Cucchetti A, Piscaglia F, Carpenzano M, Peri E, Ravaioli M, D'Errico-Grigioni A, Pinna AD, Bolondi L. Efficacy of selective transarterial chemoembolization in inducing tumor necrosis in small (& lt; 5 cm) hepatocellular carcinomas. *Hepatology* 2011; **53**: 1580-1589 [PMID: 21351114 DOI: 10.1002/hep.24246]
66 **Kwan SW**, Fidelman N, Ma E, Kerlan RK, Yao FY. Imaging predictors of the response to transarterial chemoembolization in patients with hepatocellular carcinoma: a radiological-pathological correlation. *Liver Transpl* 2012; **18**: 727-736 [PMID: 22344899 DOI: 10.1002/lt.23413]
67 **Tsochatzis E**, Garcovich M, Marelli L, Papastergiou V, Fatourou E, Rodriguez-Peralvarez ML, Germani G, Davies N, Yu D, Luong TV, Dhillon AP, Thorburn D, Patch D, O' Beirne J, Meyer T, Burroughs AK. Transarterial embolization as neo-adjuvant therapy pretransplantation in patients with hepatocellular carcinoma. *Liver Int* 2013; **33**: 944-949 [PMID: 23530918 DOI: 10.1111/liv.12144]
68 **Nicolini A**, Martinetti L, Crespi S, Maggioni M, Sangiovanni A. Transarterial chemoembolization with epirubicin-eluting beads versus transarterial embolization before liver transplantation for hepatocellular carcinoma. *J Vasc Interv Radiol* 2010; **21**: 327-332 [PMID: 20097098 DOI: 10.1016/j.jvir.2009.10.038]
69 **Majno P**, Giostra E, Mentha G. Management of hepatocellular carcinoma on the waiting list before liver transplantation: time for controlled trials? *Liver Transpl* 2007; **13**: S27-S35 [PMID: 17969086 DOI: 10.1002/lt.21328]
70 **Richard HM**, Silberzweig JE, Mitty HA, Lou WY, Ahn J, Cooper JM. Hepatic arterial complications in liver transplant recipients treated with pretransplantation chemoembolization for hepatocellular carcinoma. *Radiology* 2000; **214**: 775-779 [PMID: 10715045]
71 **Pérez Saborido B**, Meneu JC, Moreno E, García I, Moreno A, Fundora Y. Is transarterial chemoembolization necessary before liver transplantation for hepatocellular carcinoma? *Am J Surg* 2005; **190**: 383-387 [PMID: 16105523 DOI: 10.1016/j.amjsurg.2005.06.001]
72 **Shibata T**, Shibata T, Maetani Y, Isoda H, Hiraoka M. Radiofrequency ablation for small hepatocellular carcinoma: prospective comparison of internally cooled electrode and expandable electrode. *Radiology* 2006; **238**: 346-353 [PMID: 16373776 DOI: 10.1148/radiol.2381041848]
73 **Mazzaferro V**, Battiston C, Perrone S, Pulvirenti A, Regalia E, Romito R, Sarli D, Schiavo M, Garbagnati F, Marchianò A, Spreafico C, Camerini T, Mariani L, Miceli R, Andreola S. Radiofrequency ablation of small hepatocellular carci-

noma in cirrhotic patients awaiting liver transplantation: a prospective study. *Ann Surg* 2004; **240**: 900-909 [PMID: 15492574 DOI: 10.1097/01.sla.0000143301.56154.95]
74 **Lu DS**, Yu NC, Raman SS, Lassman C, Tong MJ, Britten C, Durazo F, Saab S, Han S, Finn R, Hiatt JR, Busuttil RW. Percutaneous radiofrequency ablation of hepatocellular carcinoma as a bridge to liver transplantation. *Hepatology* 2005; **41**: 1130-1137 [PMID: 15841454 DOI: 10.1002/hep.20688]
75 **Pompili M**, Mirante VG, Rondinara G, Fassati LR, Piscaglia F, Agnes S, Covino M, Ravaioli M, Fagiuoli S, Gasbarrini G, Rapaccini GL. Percutaneous ablation procedures in cirrhotic patients with hepatocellular carcinoma submitted to liver transplantation: Assessment of efficacy at explant analysis and of safety for tumor recurrence. *Liver Transpl* 2005; **11**: 1117-1126 [PMID: 16123960 DOI: 10.1002/lt.20469]
76 **Brillet PY**, Paradis V, Brancatelli G, Rangheard AS, Consigny Y, Plessier A, Durand F, Belghiti J, Sommacale D, Vilgrain V. Percutaneous radiofrequency ablation for hepatocellular carcinoma before liver transplantation: a prospective study with histopathologic comparison. *AJR Am J Roentgenol* 2006; **186**: S296-S305 [PMID: 16632691 DOI: 10.2214/AJR.04.1927]
77 **Rodríguez-Sanjuán JC**, González F, Juanco C, Herrera LA, López-Bautista M, González-Noriega M, García-Somacarrera E, Figols J, Gómez-Fleitas M, Silván M. Radiological and pathological assessment of hepatocellular carcinoma response to radiofrequency. A study on removed liver after transplantation. *World J Surg* 2008; **32**: 1489-1494 [PMID: 18373117 DOI: 10.1007/s00268-008-9559-z]
78 **DuBay DA**, Sandroussi C, Kachura JR, Ho CS, Beecroft JR, Vollmer CM, Ghanekar A, Guba M, Cattral MS, McGilvray ID, Grant DR, Greig PD. Radiofrequency ablation of hepatocellular carcinoma as a bridge to liver transplantation. *HPB* (Oxford) 2011; **13**: 24-32 [PMID: 21159100 DOI: 10.1111/j.1477-2574.2010.00228.x]
79 **Porrett PM**, Peterman H, Rosen M, Sonnad S, Soulen M, Markmann JF, Shaked A, Furth E, Reddy KR, Olthoff K. Lack of benefit of pre-transplant locoregional hepatic therapy for hepatocellular cancer in the current MELD era. *Liver Transpl* 2006; **12**: 665-673 [PMID: 16482577 DOI: 10.1002/lt.20636]
80 **Lopez KT**, Kuwada SK, Wong LL. Consequences of needle tract seeding of hepatocellular cancer after liver transplant. *Clin Transplant* 2013; **27**: E400-E406 [PMID: 23837571 DOI: 10.1111/ctr.12160]
81 **Castroagudín JF**, Delgado M, Villanueva A, Bustamante M, Martínez J, Otero E, Tomé S, Martínez SM, Segade FR, Conde R, Dominguez-Muñoz E, Varo E. Safety of percutaneous ethanol injection as neoadjuvant therapy for hepatocellular carcinoma in waiting list liver transplant candidates. *Transplant Proc* 2005; **37**: 3871-3873 [PMID: 16386568 DOI: 10.1016/j.transproceed.2005.09.168]
82 **Branco F**, Brú C, Vilana R, Bianchi L, Alves de Mattos A. Percutaneous ethanol injection before liver transplantation in the hepatocellular carcinoma. *Ann Hepatol* 2009; **8**: 220-227 [PMID: 19841501]
83 **Pacella CM**, Bizzarri G, Francica G, Forlini G, Petrolati A, Valle D, Anelli V, Bianchini A, Nuntis SD, Pacella S, Rossi Z, Osborn J, Stasi R. Analysis of factors predicting survival in patients with hepatocellular carcinoma treated with percutaneous laser ablation. *J Hepatol* 2006; **44**: 902-909 [PMID: 16545480 DOI: 10.1016/j.jhep.2006.01.031]
84 **Gough-Palmer AL**, Gedroyc WM. Laser ablation of hepatocellular carcinoma--a review. *World J Gastroenterol* 2008; **14**: 7170-7174 [PMID: 19084930 DOI: 10.3748/wjg.14.7170]
85 **Pompili M**, Pacella CM, Francica G, Angelico M, Tisone G, Craboledda P, Nicolardi E, Rapaccini GL, Gasbarrini G. Percutaneous laser ablation of hepatocellular carcinoma in patients with liver cirrhosis awaiting liver transplantation. *Eur J Radiol* 2010; **74**: e6-e11 [PMID: 19345541 DOI: 10.1016/j.ejrad.2009.03.012]
86 **Francica G**, Petrolati A, Di Stasio E, Pacella S, Stasi R, Pacella CM. Effectiveness, safety, and local progression after percutaneous laser ablation for hepatocellular carcinoma nodules up to 4 cm are not affected by tumor location. *AJR Am J Roentgenol* 2012; **199**: 1393-1401 [PMID: 23169736 DOI: 10.2214/AJR.11.7850]
87 **Lu MD**, Xu HX, Xie XY, Yin XY, Chen JW, Kuang M, Xu ZF, Liu GJ, Zheng YL. Percutaneous microwave and radiofrequency ablation for hepatocellular carcinoma: a retrospective comparative study. *J Gastroenterol* 2005; **40**: 1054-1060 [PMID: 16322950 DOI: 10.1007/s00535-005-1671-3]
88 **Boutros C**, Somasundar P, Garrean S, Saied A, Espat NJ. Microwave coagulation therapy for hepatic tumors: review of the literature and critical analysis. *Surg Oncol* 2010; **19**: e22-e32 [PMID: 19268571 DOI: 10.1016/j.suronc.2009.02.001]
89 **Zanus G**, Boetto R, Gringeri E, Vitale A, D'Amico F, Carraro A, Bassi D, Bonsignore P, Noaro G, Mescoli C, Rugge M, Angeli P, Senzolo M, Burra P, Feltracco P, Cillo U. Microwave thermal ablation for hepatocarcinoma: six liver transplantation cases. *Transplant Proc* 2011; **43**: 1091-1094 [PMID: 21620060 DOI: 10.1016/j.transproceed.2011.02.044]
90 **Riaz A**, Kulik L, Lewandowski RJ, Ryu RK, Giakoumis Spear G, Mulcahy MF, Abecassis M, Baker T, Gates V, Nayar R, Miller FH, Sato KT, Omary RA, Salem R. Radiologic-pathologic correlation of hepatocellular carcinoma treated with internal radiation using yttrium-90 microspheres. *Hepatology* 2009; **49**: 1185-1193 [PMID: 19133645 DOI: 10.1002/hep.22747]
91 **Sandroussi C**, Dawson LA, Lee M, Guindi M, Fischer S, Ghanekar A, Cattral MS, McGilvray ID, Levy GA, Renner E, Greig PD, Grant DR. Radiotherapy as a bridge to liver transplantation for hepatocellular carcinoma. *Transpl Int* 2010; **23**: 299-306 [PMID: 19843294 DOI: 10.1111/j.1432-2277.2009.00980.x]
92 **Rossi S**, Garbagnati F, Lencioni R, Allgaier HP, Marchianò A, Fornari F, Quaretti P, Tolla GD, Ambrosi C, Mazzaferro V, Blum HE, Bartolozzi C. Percutaneous radio-frequency thermal ablation of nonresectable hepatocellular carcinoma after occlusion of tumor blood supply. *Radiology* 2000; **217**: 119-126 [PMID: 11012432]
93 **Veltri A**, Moretto P, Doriguzzi A, Pagano E, Carrara G, Gandini G. Radiofrequency thermal ablation (RFA) after transarterial chemoembolization (TACE) as a combined therapy for unresectable non-early hepatocellular carcinoma (HCC). *Eur Radiol* 2006; **16**: 661-669 [PMID: 16228211 DOI: 10.1007/s00330-005-0029-9]
94 **Ashoori N**, Paprottka P, Trumm C, Bamberg F, Kolligs FT, Rentsch M, Reiser MF, Jakobs TF. Multimodality treatment with conventional transcatheter arterial chemoembolization and radiofrequency ablation for unresectable hepatocellular carcinoma. *Digestion* 2012; **85**: 18-26 [PMID: 22156507 DOI: 10.1159/000334714]
95 **Iezzi R**, Cesario V, Siciliani L, Campanale M, De Gaetano AM, Siciliano M, Agnes S, Giuliante F, Grieco A, Pompili M, Rapaccini GL, Gasbarrini A, Bonomo L. Single-step multimodal locoregional treatment for unresectable hepatocellular carcinoma: balloon-occluded percutaneous radiofrequency thermal ablation (BO-RFA) plus transcatheter arterial chemoembolization (TACE). *Radiol Med* 2013; **118**: 555-569 [PMID: 23358819 DOI: 10.1007/s11547-012-0914-7]
96 **Ashoori N**, Bamberg F, Paprottka P, Rentsch M, Kolligs FT, Siegert S, Peporte A, Al-Tubaikh JA, D'Anastasi M, Hoffmann RT, Reiser MF, Jakobs TF. Multimodality treatment for early-stage hepatocellular carcinoma: a bridging therapy for liver transplantation. *Digestion* 2012; **86**: 338-348 [PMID: 23207185 DOI: 10.1159/000342813]
97 **Lesurtel M**, Müllhaupt B, Pestalozzi BC, Pfammatter T, Clavien PA. Transarterial chemoembolization as a bridge to liver transplantation for hepatocellular carcinoma: an

evidence-based analysis. *Am J Transplant* 2006; **6**: 2644-2650 [PMID: 16939518 DOI: 10.1111/j.1600-6143.2006.01509.x]
98 **Maddala YK**, Stadheim L, Andrews JC, Burgart LJ, Rosen CB, Kremers WK, Gores G. Drop-out rates of patients with hepatocellular cancer listed for liver transplantation: outcome with chemoembolization. *Liver Transpl* 2004; **10**: 449-455 [PMID: 15004776 DOI: 10.1002/lt.20099]
99 **Fontana RJ**, Hamidullah H, Nghiem H, Greenson JK, Hussain H, Marrero J, Rudich S, McClure LA, Arenas J. Percutaneous radiofrequency thermal ablation of hepatocellular carcinoma: a safe and effective bridge to liver transplantation. *Liver Transpl* 2002; **8**: 1165-1174 [PMID: 12474157 DOI: 10.1053/jlts.2002.36394]
100 **Ciccarelli O**, Lai Q, Goffette P, Finet P, De Reyck C, Roggen F, Sempoux C, Doffagne E, Reding R, Lerut J. Liver transplantation for hepatocellular cancer: UCL experience in 137 adult cirrhotic patients. α-foetoprotein level and locoregional treatment as refined selection criteria. *Transpl Int* 2012; **25**: 867-875 [PMID: 22716073 DOI: 10.1111/j.1432-2277.2012.01512.x]
101 **Kornberg A**, Witt U, Matevossian E, Küpper B, Assfalg V, Drzezga A, Hüser N, Wildgruber M, Friess H. Extended postinterventional tumor necrosis-implication for outcome in liver transplant patients with advanced HCC. *PLoS One* 2013; **8**: e53960 [PMID: 23349774 DOI: 10.1371/journal.pone.0053960]
102 **Bharat A**, Brown DB, Crippin JS, Gould JE, Lowell JA, Shenoy S, Desai NM, Chapman WC. Pre-liver transplantation locoregional adjuvant therapy for hepatocellular carcinoma as a strategy to improve longterm survival. *J Am Coll Surg* 2006; **203**: 411-420 [PMID: 17000383 DOI: 10.1016/j.jamcollsurg.2006.06.016]
103 **Lao OB**, Weissman J, Perkins JD. Pre-transplant therapy for hepatocellular carcinoma is associated with a lower recurrence after liver transplantation. *Clin Transplant* 2009; **23**: 874-881 [PMID: 19453644 DOI: 10.1111/j.1399-0012.2009.00993.x]
104 **Heckman JT**, Devera MB, Marsh JW, Fontes P, Amesur NB, Holloway SE, Nalesnik M, Geller DA, Steel JL, Gamblin TC. Bridging locoregional therapy for hepatocellular carcinoma prior to liver transplantation. *Ann Surg Oncol* 2008; **15**: 3169-3177 [PMID: 18696158 DOI: 10.1245/s10434-008-0071-3]
105 **Freeman RB**, Steffick DE, Guidinger MK, Farmer DG, Berg CL, Merion RM. Liver and intestine transplantation in the United States, 1997-2006. *Am J Transplant* 2008; **8**: 958-976 [PMID: 18336699 DOI: 10.1111/j.1600-6143.2008.02174.x]
106 **Yao FY**, Kerlan RK, Hirose R, Davern TJ, Bass NM, Feng S, Peters M, Terrault N, Freise CE, Ascher NL, Roberts JP. Excellent outcome following down-staging of hepatocellular carcinoma prior to liver transplantation: an intention-to-treat analysis. *Hepatology* 2008; **48**: 819-827 [PMID: 18688876 DOI: 10.1002/hep.22412]
107 **De Luna W**, Sze DY, Ahmed A, Ha BY, Ayoub W, Keeffe EB, Cooper A, Esquivel C, Nguyen MH. Transarterial chemoinfusion for hepatocellular carcinoma as downstaging therapy and a bridge toward liver transplantation. *Am J Transplant* 2009; **9**: 1158-1168 [PMID: 19344435 DOI: 10.1111/j.1600-6143.2009.02576.x]
108 **Chapman WC**, Majella Doyle MB, Stuart JE, Vachharajani N, Crippin JS, Anderson CD, Lowell JA, Shenoy S, Darcy MD, Brown DB. Outcomes of neoadjuvant transarterial chemoembolization to downstage hepatocellular carcinoma before liver transplantation. *Ann Surg* 2008; **248**: 617-625 [PMID: 18936575 DOI: 10.1097/SLA.0b013e31818a07d4]
109 **Lewandowski RJ**, Kulik LM, Riaz A, Senthilnathan S, Mulcahy MF, Ryu RK, Ibrahim SM, Sato KT, Baker T, Miller FH, Omary R, Abecassis M, Salem R. A comparative analysis of transarterial downstaging for hepatocellular carcinoma: chemoembolization versus radioembolization. *Am J Transplant* 2009; **9**: 1920-1928 [PMID: 19552767 DOI: 10.1111/j.1600-6143.2009.02695.x]
110 **Jang JW**, You CR, Kim CW, Bae SH, Yoon SK, Yoo YK, Kim DG, Choi JY. Benefit of downsizing hepatocellular carcinoma in a liver transplant population. *Aliment Pharmacol Ther* 2010; **31**: 415-423 [PMID: 19821808 DOI: 10.1111/j.1365-2036.2009.04167.x]
111 **Barakat O**, Wood RP, Ozaki CF, Ankoma-Sey V, Galati J, Skolkin M, Toombs B, Round M, Moore W, Mieles L. Morphological features of advanced hepatocellular carcinoma as a predictor of downstaging and liver transplantation: an intention-to-treat analysis. *Liver Transpl* 2010; **16**: 289-299 [PMID: 20209588 DOI: 10.1002/lt.21994]
112 **Bargellini I**, Vignali C, Cioni R, Petruzzi P, Cicorelli A, Campani D, De Simone P, Filipponi F, Bartolozzi C. Hepatocellular carcinoma: CT for tumor response after transarterial chemoembolization in patients exceeding Milan criteria--selection parameter for liver transplantation. *Radiology* 2010; **255**: 289-300 [PMID: 20308465 DOI: 10.1148/radiol.09090927]
113 **Bova V**, Miraglia R, Maruzzelli L, Vizzini GB, Luca A. Predictive factors of downstaging of hepatocellular carcinoma beyond the Milan criteria treated with intra-arterial therapies. *Cardiovasc Intervent Radiol* 2013; **36**: 433-439 [PMID: 22864644 DOI: 10.1007/s00270-012-0458-1]
114 **Lei J**, Wang W, Yan L. Downstaging advanced hepatocellular carcinoma to the milan criteria may provide a comparable outcome to conventional Milan criteria. *J Gastrointest Surg* 2013; **17**: 1440-1446 [PMID: 23719776 DOI: 10.1007/s11605-013-2229-y]
115 **Ravaioli M**, Grazi GL, Piscaglia F, Trevisani F, Cescon M, Ercolani G, Vivarelli M, Golfieri R, D'Errico Grigioni A, Panzini I, Morelli C, Bernardi M, Bolondi L, Pinna AD. Liver transplantation for hepatocellular carcinoma: results of down-staging in patients initially outside the Milan selection criteria. *Am J Transplant* 2008; **8**: 2547-2557 [PMID: 19032223 DOI: 10.1111/j.1600-6143.2008.02409.x]
116 **Otto G**, Herber S, Heise M, Lohse AW, Mönch C, Bittinger F, Hoppe-Lotichius M, Schuchmann M, Victor A, Pitton M. Response to transarterial chemoembolization as a biological selection criterion for liver transplantation in hepatocellular carcinoma. *Liver Transpl* 2006; **12**: 1260-1267 [PMID: 16826556 DOI: 10.1002/lt.20837]
117 **DuBay D**, Sandroussi C, Sandhu L, Cleary S, Guba M, Cattral MS, McGilvray I, Ghanekar A, Selzner M, Greig PD, Grant DR. Liver transplantation for advanced hepatocellular carcinoma using poor tumor differentiation on biopsy as an exclusion criterion. *Ann Surg* 2011; **253**: 166-172 [PMID: 21294289 DOI: 10.1097/SLA.0b013e31820508f1]
118 **Lencioni R**, Llovet JM. Modified RECIST (mRECIST) assessment for hepatocellular carcinoma. *Semin Liver Dis* 2010; **30**: 52-60 [PMID: 20175033 DOI: 10.1055/s-0030-1247132]
119 **Gordon-Weeks AN**, Snaith A, Petrinic T, Friend PJ, Burls A, Silva MA. Systematic review of outcome of downstaging hepatocellular cancer before liver transplantation in patients outside the Milan criteria. *Br J Surg* 2011; **98**: 1201-1208 [PMID: 21618496 DOI: 10.1002/bjs.7561]
120 **Belghiti J**, Carr BI, Greig PD, Lencioni R, Poon RT. Treatment before liver transplantation for HCC. *Ann Surg Oncol* 2008; **15**: 993-1000 [PMID: 18236111 DOI: 10.1245/s10434-007-9787-8]
121 **Bruix J**, Sherman M. Management of hepatocellular carcinoma: an update. *Hepatology* 2011; **53**: 1020-1022 [PMID: 21374666 DOI: 10.1002/hep.24199]
122 **European Association For The Study Of The Liver**; European Organisation For Research And Treatment Of Cancer. EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. *J Hepatol* 2012; **56**: 908-943 [PMID: 22424438]
123 **Clavien PA**, Lesurtel M, Bossuyt PM, Gores GJ, Langer B, Perrier A. Recommendations for liver transplantation for hepatocellular carcinoma: an international consensus conference report. *Lancet Oncol* 2012; **13**: e11-e22 [PMID: 22047762 DOI: 10.1016/S1470-2045(11)70175-9]
124 **Therasse P**, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom

AT, Christian MC, Gwyther SG. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000; **92**: 205-216 [PMID: 10655437 DOI: 10.1093/jnci/92.3.205]

125 **Llovet JM**, Di Bisceglie AM, Bruix J, Kramer BS, Lencioni R, Zhu AX, Sherman M, Schwartz M, Lotze M, Talwalkar J, Gores GJ. Design and endpoints of clinical trials in hepatocellular carcinoma. *J Natl Cancer Inst* 2008; **100**: 698-711 [PMID: 18477802 DOI: 10.1093/jnci/djn134]

126 **Kim YS**, Rhim H, Lim HK, Park CK, Lee WJ, Do YS, Cho JW. Completeness of treatment in hepatocellular carcinomas treated with image-guided tumor therapies: Evaluation of positive predictive value of contrast-enhanced CT with histopathologic correlation in the explanted liver specimen. *J Comput Assist Tomogr* 2006; **30**: 578-582 [PMID: 16845287 DOI: 10.1097/00004728-200607000-00005]

127 **Bargellini I**, Bozzi E, Campani D, Carrai P, De Simone P, Pollina L, Cioni R, Filipponi F, Bartolozzi C. Modified RECIST to assess tumor response after transarterial chemoembolization of hepatocellular carcinoma: CT-pathologic correlation in 178 liver explants. *Eur J Radiol* 2013; **82**: e212-e218 [PMID: 23332890 DOI: 10.1016/j.ejrad.2012.12.009]

128 **Roberts JP**, Venook A, Kerlan R, Yao F. Hepatocellular carcinoma: Ablate and wait versus rapid transplantation. *Liver Transpl* 2010; **16**: 925-929 [PMID: 20658555 DOI: 10.1002/lt.22103]

P- Reviewers: Cheung R, Kakizaki S, Tsuchiya A
S- Editor: Cui XM **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.v19.i43.7531

World J Gastroenterol 2013 November 21; 19(43): 7531-7543
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Enteric bacterial proteases in inflammatory bowel disease-pathophysiology and clinical implications

Ian M Carroll, Nitsan Maharshak

Ian M Carroll, Division of Gastroenterology and Hepatology, Center for Gastrointestinal Biology and Disease, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, United States
Nitsan Maharshak, Department of Gastroenterology and Liver Disease, Tel Aviv Medical Center, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69643, Israel
Author contributions: Carroll IM and Maharshak N contributed equally to this work, reviewed the literature, wrote and drafted the paper.
Supported by The national institutes of health (DK092330) to Carroll IM
Correspondence to: Nitsan Maharshak, MD, Assistant Professor of Medicine, IBD Center, Department of Gastroenterology and Liver Disease, Tel Aviv Medical Center, Sackler School of Medicine, Tel Aviv University, 6 Weizmann St., Tel Aviv 69643, Israel. nitsanm@tlvmc.gov.il
Telephone: +972-3-6947304 Fax: +972-3-6974622
Received: September 17, 2013 Revised: October 15, 2013
Accepted: November 3, 2013
Published online: November 21, 2013

Abstract

Numerous reports have identified a dysbiosis in the intestinal microbiota in patients suffering from inflammatory bowel diseases (IBD), yet the mechanism(s) in which this complex microbial community initiates or perpetuates inflammation remains unclear. The purpose of this review is to present evidence for one such mechanism that implicates enteric microbial derived proteases in the pathogenesis of IBD. We highlight and discuss studies demonstrating that proteases and protease receptors are abundant in the digestive system. Additionally, we investigate studies demonstrating an association between increased luminal protease activity and activation of protease receptors, ultimately resulting in increased intestinal permeability and exacerbation of colitis in animal models as well as in human IBD. Proteases are essential for the normal functioning of bacteria and in some cases can serve as virulence factors for pathogenic bacteria. Although not classified as traditional virulence factors, proteases originating from commensal enteric bacteria also have a potential association with intestinal inflammation *via* increased enteric permeability. Reports of increased protease activity in stools from IBD patients support a possible mechanism for a dysbiotic enteric microbiota in IBD. A better understanding of these pathways and characterization of the enteric bacteria involved, their proteases, and protease receptors may pave the way for new therapeutic approaches for these diseases.

Key words: Protease; Proteinase; Protease associated receptor; Enteric microbiota; Epithelial barrier

Core tip: It is currently accepted that an enteric dysbiosis (alteration of the normal bacterial flora) is involved in the pathophysiology of inflammatory bowel diseases (IBD). One of the suggested mechanisms that ties an intestinal dysbiosis to the pathophysiology of IBD involves the release of enteric bacterial proteases that interact with protease activated receptors on epithelial cells, resulting in intestinal barrier dysfunction and exposure of the enteric immune system to luminal antigens. We have reviewed the literature that examined the role of microbial proteases and their enteric receptors in the pathogenesis of IBD, their suggested pathways of action, and discuss future therapeutic implications.

Carroll IM, Maharshak N. Enteric bacterial proteases in inflammatory bowel disease- pathophysiology and clinical implications. *World J Gastroenterol* 2013; 19(43): 7531-7543 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7531.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7531

INTRODUCTION

Inflammatory bowel diseases (IBD), collectively known as Crohn's disease (CD) and ulcerative colitis (UC), are caused by dysregulated immune responses towards microbial antigens in a genetically predisposed host. The incidence of UC and CD has been increasing worldwide in developed and in developing countries[1,2]. These diseases are highly prevalent in the United States affecting 1.4 million individuals[3] and are associated with reduced quality of life[4,5], and psychological co-morbidity[6]. Current estimates for IBD associated treatment costs in the US are $6.3 billion[7], but the initiating events of IBD and causes of disease exacerbation remain unclear. It is postulated that one potential mechanism involves disruption of the epithelial barrier, and exposure of a genetically defective immune system to enteric microbial antigens. Consistent with this hypothesis are animal models of colitis that use chemical disruption of the epithelial barrier with trinitrobenzene sulphonic acid (TNBS), dextran sodium sulfate or non-steroidal anti-inflammatory drugs (NSAIDs). Additionally, disruption of the intestinal epithelial barrier by exposure of susceptible patients to NSAIDs (blockers of prostaglandins synthesis) is a known risk factor that can trigger intestinal inflammation[8]. In line with this observation, in animal studies, the use of a prostaglandin receptor agonist preserved the intestinal epithelial barrier structure and function, maintained mucous secretion by goblet cells, and prevented the development of colitis[9].

Proteases, peptidases, or proteolytic enzymes, are a class of enzyme that catalyze the cleavage of peptide bonds in other proteins in the presence of H_2O (hydrolysis). Proteases act as both positive and negative effectors of several biological processes either broadly as catalysts of protein degradation or specifically as selective agents that control physiological processes[10]. The importance of proteases is highlighted in the human genome where 2%-4% of genes encompass the *degradome*[11]. In bacteria, proteases are involved in numerous biological processes, such as those associated with metabolism, development, and virulence. Additionally, these enzymes can disrupt mucosal barriers, provide a metabolic advantage, and modulate the host immune response. The high prevalence of proteases in enterobacteria suggests that proteases play important roles in pathogenesis[12]. Both mammalian and bacterial proteases have been implicated in the pathogenesis of IBD, usually through disruption of the epithelial barrier. In pathogenic bacteria, many proteases are virulence factors that aid in bacterial invasion into host cells and cause infectious colitis. However, accumulating evidence shows that commensal enteric microorganisms also produce proteases that possess the ability to disrupt the epithelial barrier[13,14]. These commensal proteases may be involved in the pathogenesis of IBD in the context of a genetically predisposed host and/or when an intestinal microbial dysbiosis occurs. Our aim in this review is to provide an overview of current studies that suggest potential mechanisms in which microbial proteases may play a role in the pathogenesis of IBD.

PROTEASE CLASSIFICATION

Proteases frequently exist as multi-domain proteins, with catalytic activity restricted to a single structural domain. Although these enzymes appear to have a specific function (*i.e.*, hydrolysis of proteins), they exhibit vast diversity in their action and structure and are not easily categorized by general systems of enzyme nomenclature. Thus, proteases are broadly subdivided into two major groups, exopeptidases and endopeptidases. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whilst endopeptidases cleave peptide bonds distant from the termini of the substrate. Proteases are further classified into five distinct groups on the basis of the chemical nature of the groups responsible for their catalytic activity, namely; aspartic, cysteine/thiol, metallo-, serine, and unidentified proteases[15]. In order to generate a comprehensive classification system for proteases, Rawlings and Barrett[16] developed a method to classify this group of enzymes based on the type of reaction they catalyze, the chemical nature of their catalytic site, and their evolutionary structure. This approach is a hierarchical system where classification levels were summarized as peptidases (*i.e.*, serine proteases), families and clans. This system initially recognized 84 families of proteases; however the subsequent massive accumulation of amino acid sequence data and three-dimensional structures of proteases from the scientific community warranted an updated classification system that was easily accessed for academic studies. Thus, based on the system outlined by Rawlings and Barrett[16] the MEROPS database was developed[17]. Along with data regarding protease classification, the MEROPS database also provides information regarding classification of protein inhibitors of peptidases[18], small-molecule inhibitors[19], and a collection of known protease cleavage sites and substrates[20].

Microbial proteases

Proteases are found in all forms of life suggesting that they are vital for the survival of all organisms. Micro-organisms produce a vast array of aspartic, cysteine, metallo-, and serine proteases. Microbial aspartic proteases are specific for aromatic or bulky amino acid residues on both sides of a peptide bond. They are broadly divided into two groups: pepsin- and rennin-like enzymes. Cysteine proteases generally are only active in the presence of reducing agents. Some bacterial cysteine proteases are notable for their role in virulence and the inflammatory response they illicit[21]. Metalloproteases are characterized by the requirement for a divalent metal ion for their activity. These proteases are summarized into neutral and alkaline groups based on their specificity of action[22]. Serine proteases are characterized by the presence of a serine group in their active site and have broad substrate specificity. The complex microbial community in the human gut (referred to as the intestinal microbiota) is a substantial source of serine, cysteine, and metallo-proteases[23-25]. This is exemplified by the reduction of colonic bacteria

densities and protease activity by oral administration of antibiotics to mice[26]. By analyzing the protease activity of representative enteric bacterial strains and human fecal samples it has previously been suggested that the activity of specific classes of proteases present in human feces are likely to originate from *Bacteroides*, *Streptococcus*, and *Clostridium* species[27]. However, to date only one study has reported the correlation between specific groups of proteases and the abundance of enteric bacterial taxa using modern molecular methods. Carroll *et al*[28] used high throughput sequencing of the 16S rRNA gene and correlated the abundances of specific bacterial families with fecal tryptic activity in stool samples from healthy individuals and IBS patients. This study found positive associations between *Lachnospiraceae*, *Streptococcaceae* and *Lactobacillales* with fecal protease activity, and a negative correlation with *Ruminococcaceae*. However, to date microbial proteases have been mainly exploited for commercial purposes. For example, bacterial alkaline proteases are characterized by their high activity at an alkaline pH and their broad substrate specificity, thus, making them ideal for use in the detergent industry[29]. In addition, most academic studies have focused on bacterial proteases as potential virulence factors in pathogenic bacteria[30]. However, little is known regarding the relationship between microbial proteases, found in or on the body, and the health of the host. Examples of such microbial proteases that are produced by enteric commensals are specified in Table 1.

MICROBIAL PROTEASES IN THE PATHOGENESIS OF IBD

The antigenic contents of the intestinal lumen are separated from underlying intestinal tissues by an epithelial barrier that is one cell thick. Pathogenic bacteria have acquired virulence factors, many of which are proteases, that disrupt this barrier and cause infection[31]. For example, the serine protease autotransporter of *Enterobacteriaceae* family are generally secreted into the external milieu and are highly prevalent among enteropathogens, including *Shigella* species and all *Escherichia coli* (*E. coli*) pathotypes[12]. As there is an established genetic component to IBD[32], it is difficult to identify microbial proteases that are potentially involved in the pathogenesis of these diseases as they would not be categorized in the same manner as traditional virulence factors. Indeed, an overproduction of microbial proteases originating for enteric commensal microbes may not have an effect on a healthy individual, but may play a role in the pathogenesis or perturbation of intestinal inflammation in a population with a genetic predisposition to IBD. Here we discuss four potential mechanisms in which microbial proteases from a non-pathogenic source (the intestinal microbiota) could contribute to the pathogenesis of IBD.

Table 1 Commensal enteric microbial protease classification and origin

Protease category	Microbial origin	Protease
Aspartic	*Candida albicans*	Secreted aspartic proteases[119]
	Pseudomonas aeruginosa	Type 4 prepilin peptidase[120]
	Methanococcus voltae	Preflagellin[121]
Cysteine	Gram positive bacteria	Sortases[122]
	Porphyromonas gingivalis	Gingipain[21]
	Staphylococcus aureus	Staphopain[123]
Metalloprotease	*Bacteroides fragilis*	Fragilysin[124]
	Enterococcus faecalis	Gelatinase[87]
	Staphylococcus epidermidis	Elastase[123]
	Clostridium perfringens	Collagenase[13]
Serine	*Helicobacter pylori*	High temperature requirement A[125]
	Bacillus subtilis	Subtilisin[126]

MICROBIAL PROTEASES AND ADHERENCE AND INVASION TO THE INTESTINAL EPITHELIUM

Bacterial adhesion to intestinal epithelial cells is believed to be one of the first steps used in the pathogenicity of many enteric pathogens. Adhesion enables a microbe to colonize the intestinal epithelium and resist exclusion from the intestine by the mechanical movement of the gut. Adherent and invasive *E. coli* (AIEC) are a group of enteric microbes that are capable of adhering to and invading intestinal epithelial cells[33]. AIECs are not classified as enteric pathogens, but exhibit some pathogenic traits in the context of IBD. For example, AIECs isolated from CD patients are able to replicate within macrophages without escaping from the phagosome and without inducing macrophage death[34]. Proteases for pathogenic bacteria play a fundamental role in adherence and invasion virulence traits. For example, enteroaggregative *E. coli* (EAEC) expresses a factor referred to as "protease involved in colonization" or Pic. Pic catalyzes gelatin degradation which can be abolished by disruption of the predicted proteolytic active site. This protease is involved in the early stages of pathogenesis and most probably promotes intestinal colonization[30,35]. Pic is also essential for biofilm formation in EAEC. The first step of biofilm formation is bacterial adherence to a surface and then intercellular aggregation. In general, intercellular aggregation is mediated *via* the proteolytic processing of bacterial aggregation proteins by means of host or bacterial proteases[36,37] ultimately resulting in a biofilm. To date the role of microbial proteases involved in the formation of biofilms in members of the intestinal microbiota have not been investigated in the context of IBD. However, the role of biofilms in AIEC virulence in IBD has begun to emerge. It was reported that biofilm formation indi-

ces were higher amongst AIEC than non-AIEC strains isolated from the intestinal mucosa of CD, UC, and non-IBD controls[38]. Additionally, the adhesion and invasion properties of AIECs correlated positively with higher biofilm formation indices. Furthermore, the σ^E factor, which up-regulates genes that encode proteases, periplasmic foldases, and chaperones in response to environmental stresses, plays a pivotal role in biofilm formation in AIECs in the context of CD[39]. Thus, proteases may be important in biofilm formation and colonization of commensal enteric bacteria and related to IBD pathogenesis.

PROTEASE RECEPTORS

Proteases can mediate their activity on mammalian cells through activation of protease receptors. Protease activated receptors (PARs) are a family of 7 transmembrane domain G-protein-coupled receptors (GPCRs) that mediate multiple responses to external stimuli, such as hemostasis, thrombosis and inflammation, and exist in four isoforms (PARs 1-4)[40-44]. PARs are activated through proteolytic cleavage of the extracellular N-terminal component of the receptor unmasking a tethered peptide ligand residue that binds with another region of the receptor causing a conformational change[45]. The result is an initiation of an intracellular signaling cascade that is diverse and includes calcium mobilization, phospholipase C-dependent production of inositol phosphates and diacylglycerol, Rho and Rac activation, mitogen-activated protein kinase signaling, and gene transcription[46]. Alternatively, PARs can be activated through peptide sequences that are homologues to the intrinsic tethered ligand. These synthetic peptides activate PARs without proteolysis of the N-terminal of the receptor in PAR1, PAR2 and PAR4 but not in PAR3[47]. The outcome of PAR activation is dependent on the type of ligand (*e.g.*, serine protease, matrix metalloprotease, plasmin, coagulation factors *etc.*), receptor type (PAR1, 2, 3 or 4) and on the type of cell which the PAR is expressed (*e.g.*, epithelial cells, platelets, nerve cells, or leukocytes). PAR activation, signaling and degradation are highly regulated by post translational modifications such as phosphorylation, glycosylation and ubiquitination (for review- Grimsey *et al*[48]). In the gastrointestinal (GI) tract, PARs are activated by endogenous proteases secreted by the pancreas (such as trypsin), by cells of the enteric wall (such as mast cells), or by the luminal enteric microbiota. Moreover, PAR expression on the gut epithelium differs between IBD patients and healthy individuals. This may be a result of the type of micro-organisms present in the GI tract and other receptors [such as toll-like receptors (TLRs)] they interact with. For example, on polymorphonuclear (PMN) cells, *Candida albicans* promoted a TLR2-dependent PAR1 activation and expression in contrast to *Aspergillus fumigatus* that suppressed TLR4-dependent PAR2 activation and expression[49]. In this regard it is important to note that endogenous host proteases are also PAR specific, *e.g.*, - thrombin activates PAR1[50], PAR3[40,43] and PAR4[44], while trypsin activates PAR2[51] and PAR4[52].

While the majority of research relating to the relationship between PARs and colitis has examined the role of endogenous activation of PARs by mammalian proteases, the interaction between the enteric microbes, PAR expression and activation and the pathophysiology of colitis have not been extensively studied. The evidence that supports these associations is summarized below.

PAR1

PAR1 has been implicated in hemostasis, platelet signaling, systemic pro-inflammatory responses (such as vasodilatation, increased vascular permeability and chemotaxis) and induction of analgesia[53,54]. PAR1 agonists induce apoptosis of intestinal epithelial cells in a caspase-3-dependent manner, with a concomitant loss of the epithelial barrier function and a consequent increase of permeability to macromolecules and bacteria[55]. PAR1 is expressed by enterocytes as well as by other cell types such as endothelial cells, enteric neurons, myocytes and immune cells[52]. The expression of PAR1 on the intestinal epithelium is linked to the presence of enteric microbiota[56], and activation of this receptor in the mouse colon leads to colitis[57,58]. In addition, PAR1 expression has been reported to be increased in colonic biopsies from IBD patients[54]. Altogether, these reports support a role for PAR1 in the pathogenesis of IBD, however it is not clear if the enteric microbiota directly activate PAR1 through release of bacterial proteases. Nonetheless, this mechanism is supported by a study investigating oral epithelial cells, where PAR1 activation by a cysteine protease released by the oral pathogen *Porphyromonas gingivalis* (*P. gingivalis*) caused an up-regulation of pro-inflammatory cytokines[59].

PAR2

The majority of evidence that points towards an association between PARs and intestinal inflammation involves PAR2. This receptor is localized to the apical and basolateral membrane[60-62] of the intestinal epithelium and can be activated by trypsin, tryptase, and bacterial proteases[63]. PAR2 is expressed in immune, stromal, endothelial, and intestinal epithelial cells and thus, PAR2-associated inflammation may be a result of multiple, systemic and local pathways. Systemically, this receptor impacts leukocytes by mediating rolling, adhesion, and extravasation[64]. When activated on sensory neurons PAR2 mediates pain and edema[65]. In the mouse colon, activation of this receptor results in colitis[60] that is significantly ameliorated in PAR2-deficient mice[60,66]. Additionally, antagonism of PAR2 (by GB88) results in amelioration of colitis in rats that is induced by either TNBS or a PAR2 agonist (SLIGRL-NH_2)[67]. Thus, most studies indicate that activation of PAR2 leads to an inflammatory response. However, a single study has reported a protective effect of daily intra colonic administration of PAR2 agonist in a TNBS colitis model in rats[68]. It is not entirely clear why PAR2 exhibits anti-inflammatory properties in this model; however it may be the result of a chronic PAR2 activation and lo-

cal desensitization, or *via* anti-inflammatory effects on macrophages[69]. Additionally, it is not clear which of the various mechanisms that have been implicated in PAR2 activation in the gut is responsible for PAR2-dependent colitis. However, it has been speculated that PAR2-mediated intestinal inflammation is a result of increased levels of PAR2 ligands in the colon of IBD patients. Indeed, in the colon of human IBD patients the natural PAR2 ligands, trypsin[70] and tryptase[71,72] are elevated compared to healthy controls. Moreover, in human IBD, PAR2 is overexpressed on mast cells[73] which have also been implicated in the pathogenesis of PAR2-mediated colitis. In non-IBD patients permeability was found to be proportional to the concentration of tryptase (naturally secreted by mast cells) added to the basolateral surface and not to the mucosal surface of mucosal biopsies[74]. These studies support the importance of mast cells in colitis *via* PAR2 activation, however enteric bacteria may also play a role in PAR2 activation in the colon through release of bacterial proteases in the gut lumen[24]. Róka *et al*[26] demonstrated increased levels of serine proteases in fecal samples from UC patients and hypothesized that these enzymes originated from luminal bacteria as it was reported that increased fecal protease activity was neither of a mast cells nor pancreatic origin. PAR2 can be activated by enteric bacteria either directly by bacterial proteases, as demonstrated in the oral epithelium by proteases of *P. gingivalis*[63] and in infectious colitis by the Toxin A of *Clostridium difficile*[75], or indirectly by bacterial-dependent induction of host proteases[76], as discussed above. Finally, it has been reported that antibiotic treatment directed at the gut microbiota resulted in reduced PAR2 expression suggesting that PAR2 is not only activated by enteric bacteria but its expression is also regulated by the presence of these microbes[77].

PAR3

The biological significance of PAR3 has not been fully delineated. Structurally, this PAR isotype does not have a C-terminal intra cytoplasmatic tail and thus cannot signal through GPCRs. However, PAR3 may serve as a cofactor or co-receptor of other PARs. In mouse platelets, PAR3 functions as a cofactor for PAR4 by presenting thrombin to low-affinity PAR4, thereby resulting in efficient receptor cleavage[78]. On endothelial cells PAR3 can regulate PAR activity by forming a heterodimer with PAR1[79]. Despite evidence of PAR3 mRNA expression in the small intestine, this receptor's relationship with intestinal inflammation and bacterial proteases are unknown[40].

PAR4

PAR4 is expressed in the small and large intestine[44] and is localized to colonocytes in rats[80]. It can be proteolytically activated by thrombin, trypsin and by the neutrophil granule protease cathepsin G[81]. Its activation induces leukocyte rolling and adherence, suggesting a pro-inflammatory role for this receptor[45,82-84]. Exposure of mouse colons to PAR4 agonists results in increased paracellular colonic permeability, suggesting that this receptor may be involved in the pathophysiology of IBD[85]. In the human colon, expression of PAR4 on epithelial cells is negligible in non-IBD patients but is significantly higher in UC patients. Interestingly, the activity of cathepsin G was increased in the feces of UC patients compared to controls and inhibition of its activity resulted in ameliorated enteric permeability[85]. Thus, cathepsin G may mediate PAR4-dependent enteric permeability in UC patients. Nevertheless, a direct effect of bacterial proteases was not examined; therefore it is still unknown whether proteases released by the enteric microbiota contribute to enteric permeability and colitis in a PAR4 dependent manner.

PROTEASES AND INTESTINAL BARRIER DISRUPTION

The intestinal epithelial barrier is made up of a single layer of cells that are tethered together *via* tight junctions and cell adhesion molecules. Enteric microbes can circumvent the defense of the intestinal epithelial barrier either directly through proteolytic degradation of cell adhesion molecules (such as E-cadherin) or indirectly by regulation of paracellular permeability *via* tight junctions. Intestinal epithelial tight junctions are composed of different protein complexes which consist of transmembrane and intracellular scaffold proteins (Figure 1).

The trans-membrane proteins include occludin, claudins, and junctional adhesion molecules whose extracellular loops are bound together and intracellular domains interact with scaffold proteins such as zonula occludens (ZO), which in turn are anchored to the actin cytoskeleton. In the intestine the adherence junction protein, E-cadherin, cements epithelial cells together and is a significant factor in maintenance of the epithelial barrier function. The enteric commensal *Enterococcus faecalis* (*E. faecalis*) can induce inflammation in a gnotobiotic *IL-10*$^{-/-}$ mouse[86] and secretes a protease (gelatinase) which has the capacity to degrade collagen, fibrinogen, fibrin, endothelin-1, bradykinin, LL-37, and complement components C3 and C3a[87-92]. The potential of *E. faecalis* gelatinase to damage the intestinal epithelial barrier and cause inflammation in the *IL-10*$^{-/-}$ mouse was recently investigated[14]. Steck and associates created an *E. faecalis* mutant lacking the *gelE* gene (Δ*gelE*). *IL-10*$^{-/-}$ mice mono-associated with *E. faecalis* Δ*gelE* exhibited significantly lower colonic inflammation when compared to mice mono-associated with wild-type *E. faecalis*. The reduction in colonic inflammation was independent of colonization densities of *E. faecalis* strains. Interestingly, the expression of E-cadherin on epithelial cells in *IL-10*$^{-/-}$ mice was reduced in the presence of gelE (wild-type *E. faecalis*) but not when gelE was absent from *E. faecalis* (Δ*gelE*). It was further demonstrated that *E. faecalis* gelE can degrade recombinant mouse E-cadherin. These data strongly suggest a mechanism in which a bacterial protease can disrupt the intestinal barrier function and lead

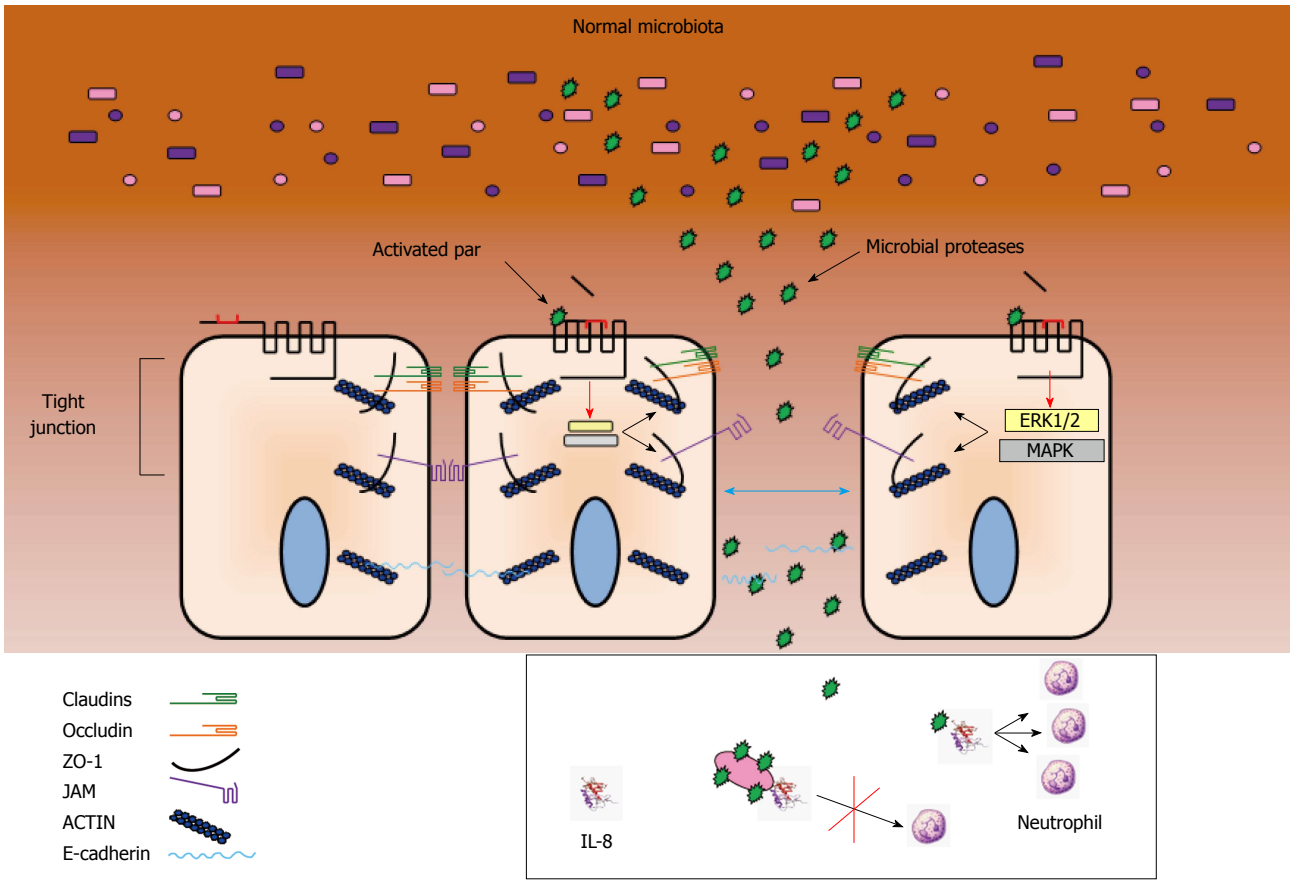


Figure 1 Model for enteric microbial protease-dependent increased intestinal permeability. Enteric microbial proteases activate epithelial protease activated receptors (PARs) through release of the tethered ligand. This results in intra-cellular signal transduction and activation of ERK 1, 2 and MAPK. These signaling molecules mediate disruption of tight junctions and consequently cause increased intestinal permeability that enables penetration of microbes and their proteases which can act upon cytokines. Further possible effects of bacterial proteases on the immune response are illustrated in the black box. These mechanisms have been demonstrated for *Porphyromonas gingivalis* in the oral cavity (and not in the gut) where gingipain proteases can enhance Interleukin (IL)-8-dependent attraction of neutrophils (when in their soluble forms) by partially degrading the N-terminal of this cytokine, or inhibit neutrophil activity via complete degradation of IL-8 when associated with the microbial membrane.

to inflammation. This finding is specifically significant to CD pathogenesis where a greater diversity of microbes with gelatinolytic activity was reported when compared to healthy controls[13].

The intestinal microbiota has long been thought of as a significant contributor to the proteolytic activity of stool[24,27]. Specifically, Macfarlane *et al*[24] found that the proteolytic activity in the stool from a patient that had undergone a pancreatectomy was comparable to that of the protease activities in stools from individuals that had not undergone surgery to remove their pancreas. This indicates that a source other than the pancreas (*i.e.*, enteric microbes) significantly contributes to the protease activity of the intestine. These observations have been more recently demonstrated by the reduction of colonic bacteria densities and protease activity by oral administration of antibiotics to mice[26]. As previously mentioned, increased protease activity has been reported in fecal samples obtained from subgroups of patients suffering from irritable bowel syndrome (IBS) and IBD[25,93,94]. Róka *et al*[26] initially saw a four-fold increase in trypsin-like activity in diarrhea-predominant IBS (D-IBS) and UC patients. Subsequently, it was found that fecal supernatants from D-IBS patients could increase colonic paracellular permeability in the mouse gut[94]. The application of D-IBS supernatants to the mouse colon resulted in an increase in phosphorylation of myosin light chain kinase and delayed redistribution of the tight junction-associated molecule ZO-1. Further investigations demonstrated that fecal supernatants from UC patients can affect visceral sensitivity and colonic permeability in mice that was mediated *via* differing protease receptors (see protease receptors in this review). Together these studies suggest a mechanism in which microbial proteases can alter intestinal barrier function by regulating tight-junctions.

ENTERIC MICROBIAL PROTEASES AND IMMUNE CELL REGULATION

Once the intestinal epithelial barrier has been breached microbes or microbial antigens can potentially traverse into the underlying tissues of the intestine and interact with immune cells, ultimately leading to inflammation. Although enteric microbes are essential environmental

factors for immune cell development, as evidenced by an under established immune system found in germ-free mice[95], the immune system can also be subverted by enteric microorganisms *via* microbial proteases. Bacterial proteases capable of disrupting cytokine signaling can potentially affect the pathogenesis of disease. For example, cysteine protease gingipains K (Kgp) and R (RgpA and RgpB) are produced by *P. gingivalis* and are significant factors in this oral microbe's pathogenesis[96]. Soluble gingipains secreted by *P. gingivalis* are capable of cleaving the N-terminus of IL-8 and enhancing this cytokine's activity of attracting neutrophils[97]. Additionally, Kgp, RgpA, and RpgB can also instantly degrade IL-8 when these enzymes are associated with membrane vesicles of *P. gingivalis*. This dual role of enhancing and inhibiting immune cell activity by the soluble and membrane-bound forms of these microbial proteases, respectively, may explain the pro- and anti-inflammatory sites found in periodontitis infections. The massive infiltration of neutrophils at periodontitis sites without the elimination of infection may also be explained by the dual roles of these microbial proteases. Another example is that of necrotizing fasciitis caused by *Streptococcus pyogenes* (*S. pyogenes*) that is characterized by an absence of neutrophils within lesions. It has been reported that the relative absence of neutrophils in necrotizing fasciitis lesions were due to restricted proteolysis of the C-terminal of IL-8 by the *S. pyogenes* protease *Sly*CEP[98]. Further investigations revealed that cleavage of the IL-8 C-terminal by *Sly*CEP from *S. pyogenes* is sufficient to reduce neutrophil endothelial trans-migration and is fundamental in the promotion of resistance of this microbe to neutrophil killing[99]. Given that a homologue of *Sly*CEP has been found in another *Streptococcus* species and no substrates other than cytokines have been identified, it is likely that this microbial protease is an effective weapon used by streptococci to impair bacterial clearance by neutrophils. Enteric microbial proteases can not only affect cytokines that are responsible for attracting the cellular branch of the innate immune system, but can also directly act upon neutrophils, macrophages, monocytes, and natural killer cells. SpeB from *S. pyogenes* has been shown to cause mitochondrial damage and prevent phagocytosis by granulocytes[100]. Additionally, a cysteine protease from *Staphylococcus aureus* (SspB) has been shown to selectively cleave CD11b on phagocytes which undergo apoptosis and are subsequently cleared by macrophages[101]. Taken together these studies identify microbial proteases from pathogenic and potentially commensal sources important molecules that have the ability to regulate the host immune system *via* specific mechanisms.

FUTURE FOR MICROBIAL PROTEASES AND IBD

The importance of the enteric microbiota in IBD has been established during the last decade[102]. Currently, efforts are being made to decipher the pathways through which bacteria and their products cross-talk with various cell types in the digestive tract that can potentially mediate inflammatory responses, pain or protection from chronic inflammation. The diversity of bacterial proteases and their effect on the intestinal epithelial, immune cells, and the enteric nervous system through various receptors open new avenues for research and potential therapeutic targets. Characterization of pathogenic proteases in IBD, the bacterial species that produce them and their mechanism of action are required to enhance our capability to understand the pathogenesis of these diseases and therapeutically intervene. Potential targets for therapeutic intervention include the following.

Specific bacterial groups that carry potentially pathogenic bacterial proteases

The list of specific enteric bacteria that carry bacterial proteases that can disrupt epithelial barrier function and cause colitis in animal models is small and has been discussed earlier in this review. In humans there is even less information. However, the beneficial effects of antibiotics and probiotics in pouchitis[103,104] and IBS[105-107], and antibiotics in CD[108] are well established. Although the proposed mechanisms for antibiotic and probiotic action are beyond the scope of this review, it is conceivable that one of the mechanisms involves action against protease-producing bacteria that cause increased permeability, pain and activation of the immune response. Future research characterizing these bacteria using high throughput sequencing, proteomics and metabolomics will potentially identify microbial targets for treatment of IBD.

Bacterial proteases

Production of proteases is not restricted to bacteria. Host derived proteases have an important role in normal physiology of the digestion, immune response, signaling *etc.* Therefore, strategies that target bacterial derived, intraluminal, colonic proteases without harming the host may prove to be beneficial. Novel drugs for IBD could potentially target bacterial protease production or secretion, such as the serine protease autotransporters from *Enterobacteriaceae*[12]. This approach was recently demonstrated by Löwer *et al*[109] who investigated a specific inhibitor for the *Helicobacter pylori* serine protease. High temperature requirement A (HtrA) is a secreted serine protease that cleaves E-cadherin on the surface of host cells and disrupts the epithelial barrier. Through a receptor-based virtual screening method, they found a specific inhibitor of HtrA activity that was able to prevent *in vitro* cleavage of E-cadherin, without cross reactivity to mammalian proteases. HtrA is a virulence factor for other enteric bacteria, such as *E. coli*, *Shigella flexneri* and *Campylobacter jejuni*[110]. Thus, examining the ability of this inhibitor to reduce HtrA activity and its effect on intestinal inflammation and permeability in models of colitis is warranted.

An alternative approach is to use probiotics that can be beneficial through various mechanisms such as favorable metabolic effects on the epithelial cells, anti-bacterial activity or directly through production of protease inhibi-

tors. For example, *Bifidobacterium longum* and *Bifidobacterium breve* produce serine protease inhibitors (serpins)[111,112] that may antagonize potentially pathogenic bacteria proteases and may exert at least part of its favorable effects on the colon through this mechanism. Another probiotic micro-organism, the yeast *Saccharomyces boulardii*, produces a serine protease that is beneficial to the host through it activity against *Clostridium difficile* adherence to the gut wall and against its toxin, and thus suppresses bacterial overgrowth and infectious colitis[113]. These examples demonstrate that the potential favorable effects of the enteric microbiota on gut inflammation are vast and involve multiple mechanisms that are not yet fully understood.

Protease activated receptors

PARs can be activated or antagonized by synthetic peptides that are analogous to the tethered ligand, irrespective of proteolytic cleavage of the receptor. Design of new, selective and potent drugs that correspond to the tethered ligand but also contain non-peptidic moieties may become useful in selective activation or inhibition of specific PARs. Activation of PAR2 is associated with colitis in animal models and has been used as a colitis model in rats[67] while oral administration of PAR2 antagonist resulted in amelioration of colitis. Although it is not clear if this action antagonizes host or bacterial derived proteases, the advantage of this approach is that it targets the final common receptor of the proteases, regardless of their source (bacterial or mammalian).

An additional approach would be to block PAR associated receptors. There is evidence that PAR signaling by *Candida* or *Aspergilus* on PMNs depends on the presence of TLR 2 and 4[49]. Although, similar studies regarding enteric epithelial cells is lacking, it is conceivable that such mechanisms are also required to induce PAR signaling on epithelial cells, and thus may be serve as additional potential targets against activation by microbial proteases.

Inhibitors of downstream molecular pathways

Activation of PARs by bacterial proteases results in diverse and complex signaling pathways. Characterization of specific pathways that may be inhibited to block the pathogenic effect of bacterial proteases without harming host homeostatic pathways, are required. Pepducins are such an evolving therapy[114]. Peducins are lipoprotein molecules, composed of a synthetic peptide sequence (10-20 amino acids) that relates to the GPCR intracellular sequences and of a lipid hydrophobic moiety. The lipid component tethers the pepducin in the lipid bilayer membrane of the cells and enables these molecules to interact with specific and stabilize GPCRs (for review, Dimond *et al*[115]). Specific pepducins that act as antagonists of PAR1 GPCR (P1pal-7) have shown favorable results in pre-clinical trials for lung cancer[116]. Additionally, PAR2 GPCR specific pepducin (P2pal-18S) ameliorates experimental pancreatitis through inhibition of PAR2 action that is expressed on pancreatic acinar cells[117] and ameliorates inflammation in additional mouse models[118].

CONCLUSION

In this review we have discussed the putative role and evidence of microbial proteases in inflammatory bowel disease pathogenesis. Proteases are essential for normal physiological development and are involved in numerous processes in our body. They are secreted by various cell types and their receptors are abundant in the gut wall, on immune cells, epithelial cells, and on neuronal cells. A growing amount of evidence supports a role for proteases and their receptors to IBD pathophysiology. The understanding that the enteric microbiota are crucial to disease initiation, and the fact that proteases are secreted by most bacteria and are considered virulence factors in infectious colitis, suggest that perhaps commensal bacterial proteases can also damage epithelial barrier function and may be involved in the initiation and perpetuation of IBD in genetically predisposed patients. Indeed, in this review we have summarized the current evidence that support this notion, the mechanisms through which bacterial proteases can impact the mucosal barrier function (through activation of PAR receptors), and the downstream signal pathways that result in increased epithelial permeability and perhaps in colitis.

However, it is not clear whether the proteolytic activity found in the gut lumen is exclusively of mammalian or bacterial origin. This is complicated by the fact that mammalian proteases, such as pancreatic digestive enzymes, are abundant in the gut lumen, and proteases secreted within the gut wall by leukocytes, such as neutrophils (cathepsin G) or mast cells (tryptase), "spill" into the inflamed gut. These factors may account for some of the discrepancies found between various studies investigating the origin of luminal proteolytic activity and the receptors they activate. Moreover, it is currently difficult to characterize luminal proteolytic activity, and while some research studies examine tryptic activity or gelatinase activity (each of which represents only a portion of the total luminal proteolytic activity) other studies have sought to characterize total luminal protease activity *via* functional assays and through inhibition of specific protease activity. These challenges may also explain why it is not fully clear which PAR isotype mediates increased enteric permeability and inflammation. For example, PAR1 and PAR2 have been implicated in mediating enteric inflammation or permeability by bacterial proteases or mammalian proteases while activation of PAR4 can equally result in increased enteric permeability. It is not improbable to hypothesize that for increased intestinal permeability and colitis to occur there is a multi-factorial hit process that results in activation of multiple PARs simultaneously by different proteases.

Only now we begin to unravel the effects of alterations in the normal enteric microbiota (dysbiosis), and how these "normal" bacteria can potentially induce colitis. The current challenge is to explore which commensal bacteria can secrete proteases that result in damage of the mucosal barrier. Additionally, we need to understand

which microbes are associated with colitis and what the genetically predisposing factors are that "allow" these events to happen. For example, genetic mutations associated with the reduction of mucus production and increased mucosal bacterial adherence, immune abnormalities that result in dysbiosis, and innate immune response defects that cause dysregulated immune responses once the mucosal barrier is breached.

Investigating these aspects through cell lines, mono-associated gnotobiotic animals, Ussing chambers, high-throughput sequencing of microbial DNA, metabolomics and genome wide association studies will enable us to understand the role of enteric microbial proteases in the pathogenesis of IBD and to develop effective targeted therapies that will involve specific enteric bacteria, PARs, and the downstream regulation and host immune response.

REFERENCES

1 **Molodecky NA**, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, Benchimol EI, Panaccione R, Ghosh S, Barkema HW, Kaplan GG. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* 2012; **142**: 46-54.e42; quiz e30 [PMID: 22001864 DOI: 10.1053/j.gastro.2011.10.001]

2 **Ng SC**, Tang W, Ching JY, Wong M, Chow CM, Hui AJ, Wong TC, Leung VK, Tsang SW, Yu HH, Li MF, Ng KK, Kamm MA, Studd C, Bell S, Leong R, de Silva HJ, Kasturiratne A, Mufeena MN, Ling KL, Ooi CJ, Tan PS, Ong D, Goh KL, Hilmi I, Pisespongsa P, Manatsathit S, Rerknimitr R, Aniwan S, Wang YF, Ouyang Q, Zeng Z, Zhu Z, Chen MH, Hu PJ, Wu K, Wang X, Simadibrata M, Abdullah M, Wu JC, Sung JJ, Chan FK. Incidence and phenotype of inflammatory bowel disease based on results from the Asia-pacific Crohn's and colitis epidemiology study. *Gastroenterology* 2013; **145**: 158-165.e2 [PMID: 23583432 DOI: 10.1053/j.gastro.2013.04.007]

3 **Sandborn WJ**, Present DH, Isaacs KL, Wolf DC, Greenberg E, Hanauer SB, Feagan BG, Mayer L, Johnson T, Galanko J, Martin C, Sandler RS. Tacrolimus for the treatment of fistulas in patients with Crohn's disease: a randomized, placebo-controlled trial. *Gastroenterology* 2003; **125**: 380-388 [PMID: 12891539]

4 **Cohen RD**. The quality of life in patients with Crohn's disease. *Aliment Pharmacol Ther* 2002; **16**: 1603-1609 [PMID: 12197839]

5 **Ferguson A**, Sedgwick DM, Drummond J. Morbidity of juvenile onset inflammatory bowel disease: effects on education and employment in early adult life. *Gut* 1994; **35**: 665-668 [PMID: 8200562]

6 **Graff LA**, Walker JR, Bernstein CN. Depression and anxiety in inflammatory bowel disease: a review of comorbidity and management. *Inflamm Bowel Dis* 2009; **15**: 1105-1118 [PMID: 19161177]

7 **Kappelman MD**, Rifas-Shiman SL, Porter CQ, Ollendorf DA, Sandler RS, Galanko JA, Finkelstein JA. Direct health care costs of Crohn's disease and ulcerative colitis in US children and adults. *Gastroenterology* 2008; **135**: 1907-1913 [PMID: 18854185]

8 **Dubeau MF**, Iacucci M, Beck PL, Moran GW, Kaplan GG, Ghosh S, Panaccione R. Drug-induced inflammatory bowel disease and IBD-like conditions. *Inflamm Bowel Dis* 2013; **19**: 445-456 [PMID: 22573536 DOI: 10.1002/ibd.22990]

9 **Jiang GL**, Nieves A, Im WB, Old DW, Dinh DT, Wheeler L. The prevention of colitis by E Prostanoid receptor 4 agonist through enhancement of epithelium survival and regeneration. *J Pharmacol Exp Ther* 2007; **320**: 22-28 [PMID: 17008451 DOI: 10.1124/jpet.106.111146]

10 **Neurath H**. Evolution of proteolytic enzymes. *Science* 1984; **224**: 350-357 [PMID: 6369538]

11 **Puente XS**, Sánchez LM, Gutiérrez-Fernández A, Velasco G, López-Otín C. A genomic view of the complexity of mammalian proteolytic systems. *Biochem Soc Trans* 2005; **33**: 331-334 [PMID: 15787599 DOI: 10.1042/BST0330331]

12 **Ruiz-Perez F**, Nataro JP. Bacterial serine proteases secreted by the autotransporter pathway: classification, specificity, and role in virulence. *Cell Mol Life Sci* 2013 May 21; Epub ahead of print [PMID: 23689588 DOI: 10.1007/s00018-013-1355-8]

13 **Pruteanu M**, Hyland NP, Clarke DJ, Kiely B, Shanahan F. Degradation of the extracellular matrix components by bacterial-derived metalloproteases: implications for inflammatory bowel diseases. *Inflamm Bowel Dis* 2011; **17**: 1189-1200 [PMID: 20853433 DOI: 10.1002/ibd.21475]

14 **Steck N**, Hoffmann M, Sava IG, Kim SC, Hahne H, Tonkonogy SL, Mair K, Krueger D, Pruteanu M, Shanahan F, Vogelmann R, Schemann M, Kuster B, Sartor RB, Haller D. Enterococcus faecalis metalloprotease compromises epithelial barrier and contributes to intestinal inflammation. *Gastroenterology* 2011; **141**: 959-971 [PMID: 21699778 DOI: 10.1053/j.gastro.2011.05.035]

15 **Barrett AJ**, Tolle DP, Rawlings ND. Managing peptidases in the genomic era. *Biol Chem* 2003; **384**: 873-882 [PMID: 12887054 DOI: 10.1515/BC.2003.098]

16 **Rawlings ND**, Barrett AJ. Evolutionary families of peptidases. *Biochem J* 1993; **290** (Pt 1): 205-218 [PMID: 8439290]

17 **Rawlings ND**, Barrett AJ. MEROPS: the peptidase database. *Nucleic Acids Res* 1999; **27**: 325-331 [PMID: 9847218]

18 **Rawlings ND**, Tolle DP, Barrett AJ. MEROPS: the peptidase database. *Nucleic Acids Res* 2004; **32**: D160-D164 [PMID: 14681384 DOI: 10.1093/nar/gkh071]

19 **Rawlings ND**, Morton FR. The MEROPS batch BLAST: a tool to detect peptidases and their non-peptidase homologues in a genome. *Biochimie* 2008; **90**: 243-259 [PMID: 17980477 DOI: 10.1016/j.biochi.2007.09.014]

20 **Rawlings ND**. A large and accurate collection of peptidase cleavages in the MEROPS database. *Database* (Oxford) 2009; **2009**: bap015 [PMID: 20157488 DOI: 10.1093/database/bap015]

21 **Oido-Mori M**, Rezzonico R, Wang PL, Kowashi Y, Dayer JM, Baehni PC, Chizzolini C. Porphyromonas gingivalis gingipain-R enhances interleukin-8 but decreases gamma interferon-inducible protein 10 production by human gingival fibroblasts in response to T-cell contact. *Infect Immun* 2001; **69**: 4493-4501 [PMID: 11401991 DOI: 10.1128/IAI.69.7.4493-4501.2001]

22 **Rawlings ND**, Barrett AJ. Evolutionary families of metallopeptidases. *Methods Enzymol* 1995; **248**: 183-228 [PMID: 7674922]

23 **Gibson SA**, McFarlan C, Hay S, MacFarlane GT. Significance of microflora in proteolysis in the colon. *Appl Environ Microbiol* 1989; **55**: 679-683 [PMID: 2648991]

24 **Macfarlane GT**, Allison C, Gibson SA, Cummings JH. Contribution of the microflora to proteolysis in the human large intestine. *J Appl Bacteriol* 1988; **64**: 37-46 [PMID: 3127369]

25 **Róka R**, Rosztóczy A, Leveque M, Izbéki F, Nagy F, Molnár T, Lonovics J, Garcia-Villar R, Fioramonti J, Wittmann T, Bueno L. A pilot study of fecal serine-protease activity: a pathophysiologic factor in diarrhea-predominant irritable bowel syndrome. *Clin Gastroenterol Hepatol* 2007; **5**: 550-555 [PMID: 17336590]

26 **Róka R**, Demaude J, Cenac N, Ferrier L, Salvador-Cartier C, Garcia-Villar R, Fioramonti J, Bueno L. Colonic luminal proteases activate colonocyte proteinase-activated receptor-2 and regulate paracellular permeability in mice. *Neurogastro-*

enterol Motil 2007; **19**: 57-65 [PMID: 17187589 DOI: 10.1111/j.1365-2982.2006.00851.x]
27 **Macfarlane GT**, Cummings JH, Allison C. Protein degradation by human intestinal bacteria. *J Gen Microbiol* 1986; **132**: 1647-1656 [PMID: 3543210]
28 **Carroll IM**, Ringel-Kulka T, Ferrier L, Wu MC, Siddle JP, Bueno L, Ringel Y. Fecal protease activity is associated with compositional alterations in the intestinal microbiota. *PLoS One* 2013; **8**: e78017 [PMID: 24147109 DOI: 10.1371/journal.pone.0078017]
29 **Gupta R**, Beg QK, Khan S, Chauhan B. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. *Appl Microbiol Biotechnol* 2002; **60**: 381-395 [PMID: 12466877 DOI: 10.1007/s00253-002-1142-1]
30 **Henderson IR**, Czeczulin J, Eslava C, Noriega F, Nataro JP. Characterization of pic, a secreted protease of Shigella flexneri and enteroaggregative Escherichia coli. *Infect Immun* 1999; **67**: 5587-5596 [PMID: 10531204]
31 **Yen YT**, Kostakioti M, Henderson IR, Stathopoulos C. Common themes and variations in serine protease autotransporters. *Trends Microbiol* 2008; **16**: 370-379 [PMID: 18595714 DOI: 10.1016/j.tim.2008.05.003]
32 **Jostins L**, Ripke S, Weersma RK, Duerr RH, McGovern DP, Hui KY, Lee JC, Schumm LP, Sharma Y, Anderson CA, Essers J, Mitrovic M, Ning K, Cleynen I, Theatre E, Spain SL, Raychaudhuri S, Goyette P, Wei Z, Abraham C, Achkar JP, Ahmad T, Amininejad L, Ananthakrishnan AN, Andersen V, Andrews JM, Baidoo L, Balschun T, Bampton PA, Bitton A, Boucher G, Brand S, Büning C, Cohain A, Cichon S, D'Amato M, De Jong D, Devaney KL, Dubinsky M, Edwards C, Ellinghaus D, Ferguson LR, Franchimont D, Fransen K, Gearry R, Georges M, Gieger C, Glas J, Haritunians T, Hart A, Hawkey C, Hedl M, Hu X, Karlsen TH, Kupcinskas L, Kugathasan S, Latiano A, Laukens D, Lawrance IC, Lees CW, Louis E, Mahy G, Mansfield J, Morgan AR, Mowat C, Newman W, Palmieri O, Ponsioen CY, Potocnik U, Prescott NJ, Regueiro M, Rotter JI, Russell RK, Sanderson JD, Sans M, Satsangi J, Schreiber S, Simms LA, Sventoraityte J, Targan SR, Taylor KD, Tremelling M, Verspaget HW, De Vos M, Wijmenga C, Wilson DC, Winkelmann J, Xavier RJ, Zeissig S, Zhang B, Zhang CK, Zhao H; International IBD Genetics Consortium (IIBDGC), Silverberg MS, Annese V, Hakonarson H, Brant SR, Radford-Smith G, Mathew CG, Rioux JD, Schadt EE, Daly MJ, Franke A, Parkes M, Vermeire S, Barrett JC, Cho JH. Host-microbe interactions have shaped the genetic architecture of inflammatory bowel disease. *Nature* 2012; **491**:119-124 [PMID: 23128233 DOI: 10.1038/nature11582]
33 **Rolhion N**, Darfeuille-Michaud A. Adherent-invasive Escherichia coli in inflammatory bowel disease. *Inflamm Bowel Dis* 2007; **13**: 1277-1283 [PMID: 17476674 DOI: 10.1002/ibd.20176]
34 **Bringer MA**, Glasser AL, Tung CH, Méresse S, Darfeuille-Michaud A. The Crohn's disease-associated adherent-invasive Escherichia coli strain LF82 replicates in mature phagolysosomes within J774 macrophages. *Cell Microbiol* 2006; **8**: 471-484 [PMID: 16469058 DOI: 10.1111/j.1462-5822.2005.00639.x]
35 **Harrington SM**, Sheikh J, Henderson IR, Ruiz-Perez F, Cohen PS, Nataro JP. The Pic protease of enteroaggregative Escherichia coli promotes intestinal colonization and growth in the presence of mucin. *Infect Immun* 2009; **77**: 2465-2473 [PMID: 19349428 DOI: 10.1128/IAI.01494-08]
36 **Hancock LE**, Perego M. The Enterococcus faecalis fsr two-component system controls biofilm development through production of gelatinase. *J Bacteriol* 2004; **186**: 5629-5639 [PMID: 15317767 DOI: 10.1128/JB.186.17.5629-5639.2004]
37 **Thomas VC**, Thurlow LR, Boyle D, Hancock LE. Regulation of autolysis-dependent extracellular DNA release by Enterococcus faecalis extracellular proteases influences biofilm development. *J Bacteriol* 2008; **190**: 5690-5698 [PMID: 18556793 DOI: 10.1128/JB.00314-08]
38 **Martinez-Medina M**, Naves P, Blanco J, Aldeguer X, Blanco JE, Blanco M, Ponte C, Soriano F, Darfeuille-Michaud A, Garcia-Gil LJ. Biofilm formation as a novel phenotypic feature of adherent-invasive Escherichia coli (AIEC). *BMC Microbiol* 2009; **9**: 202 [PMID: 19772580 DOI: 10.1186/1471-2180-9-202]
39 **Chassaing B**, Darfeuille-Michaud A. The σE pathway is involved in biofilm formation by Crohn's disease-associated adherent-invasive Escherichia coli. *J Bacteriol* 2013; **195**: 76-84 [PMID: 23104802 DOI: 10.1128/JB.01079-12]
40 **Ishihara H**, Connolly AJ, Zeng D, Kahn ML, Zheng YW, Timmons C, Tram T, Coughlin SR. Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature* 1997; **386**: 502-506 [PMID: 9087410 DOI: 10.1038/386502a0]
41 **Kahn ML**, Hammes SR, Botka C, Coughlin SR. Gene and locus structure and chromosomal localization of the protease-activated receptor gene family. *J Biol Chem* 1998; **273**: 23290-23296 [PMID: 9722561]
42 **Nystedt S**, Emilsson K, Larsson AK, Strömbeck B, Sundelin J. Molecular cloning and functional expression of the gene encoding the human proteinase-activated receptor 2. *Eur J Biochem* 1995; **232**: 84-89 [PMID: 7556175]
43 **Vu TK**, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* 1991; **64**: 1057-1068 [PMID: 1672265]
44 **Xu WF**, Andersen H, Whitmore TE, Presnell SR, Yee DP, Ching A, Gilbert T, Davie EW, Foster DC. Cloning and characterization of human protease-activated receptor 4. *Proc Natl Acad Sci USA* 1998; **95**: 6642-6646 [PMID: 9618465]
45 **Hollenberg MD**, Compton SJ. International Union of Pharmacology. XXVIII. Proteinase-activated receptors. *Pharmacol Rev* 2002; **54**: 203-217 [PMID: 12037136]
46 **Ossovskaya VS**, Bunnett NW. Protease-activated receptors: contribution to physiology and disease. *Physiol Rev* 2004; **84**: 579-621 [PMID: 15044683 DOI: 10.1152/physrev.00028.2003]
47 **Kawabata A**, Matsunami M, Sekiguchi F. Gastrointestinal roles for proteinase-activated receptors in health and disease. *Br J Pharmacol* 2008; **153** Suppl 1: S230-S240 [PMID: 17994114 DOI: 10.1038/sj.bjp.0707491]
48 **Grimsey N**, Soto AG, Trejo J. Regulation of protease-activated receptor signaling by post-translational modifications. *IUBMB Life* 2011; **63**: 403-411 [PMID: 21438117 DOI: 10.1002/iub.442]
49 **Moretti S**, Bellocchio S, Bonifazi P, Bozza S, Zelante T, Bistoni F, Romani L. The contribution of PARs to inflammation and immunity to fungi. *Mucosal Immunol* 2008; **1**: 156-168 [PMID: 19079173 DOI: 10.1038/mi.2007.13]
50 **Vu TK**, Wheaton VI, Hung DT, Charo I, Coughlin SR. Domains specifying thrombin-receptor interaction. *Nature* 1991; **353**: 674-677 [PMID: 1717851 DOI: 10.1038/353674a0]
51 **Molino M**, Woolkalis MJ, Reavey-Cantwell J, Praticó D, Andrade-Gordon P, Barnathan ES, Brass LF. Endothelial cell thrombin receptors and PAR-2. Two protease-activated receptors located in a single cellular environment. *J Biol Chem* 1997; **272**: 11133-11141 [PMID: 9111010]
52 **Déry O**, Corvera CU, Steinhoff M, Bunnett NW. Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. *Am J Physiol* 1998; **274**: C1429-C1452 [PMID: 9696685]
53 **Asfaha S**, Brussee V, Chapman K, Zochodne DW, Vergnolle N. Proteinase-activated receptor-1 agonists attenuate nociception in response to noxious stimuli. *Br J Pharmacol* 2002; **135**: 1101-1106 [PMID: 11877315 DOI: 10.1038/sj.bjp.0704568]
54 **Vergnolle N**, Cellars L, Mencarelli A, Rizzo G, Swaminathan S, Beck P, Steinhoff M, Andrade-Gordon P, Bunnett NW, Hollenberg MD, Wallace JL, Cirino G, Fiorucci S. A role for proteinase-activated receptor-1 in inflammatory

bowel diseases. *J Clin Invest* 2004; **114**: 1444-1456 [PMID: 15545995 DOI: 10.1172/JCI21689]
55 **Chin AC**, Vergnolle N, MacNaughton WK, Wallace JL, Hollenberg MD, Buret AG. Proteinase-activated receptor 1 activation induces epithelial apoptosis and increases intestinal permeability. *Proc Natl Acad Sci USA* 2003; **100**: 11104-11109 [PMID: 12960392 DOI: 10.1073/pnas.1831452100]
56 **Reinhardt C**, Bergentall M, Greiner TU, Schaffner F, Ostergren-Lundén G, Petersen LC, Ruf W, Bäckhed F. Tissue factor and PAR1 promote microbiota-induced intestinal vascular remodelling. *Nature* 2012; **483**: 627-631 [PMID: 22407318 DOI: 10.1038/nature10893]
57 **Cenac N**, Cellars L, Steinhoff M, Andrade-Gordon P, Hollenberg MD, Wallace JL, Fiorucci S, Vergnolle N. Proteinase-activated receptor-1 is an anti-inflammatory signal for colitis mediated by a type 2 immune response. *Inflamm Bowel Dis* 2005; **11**: 792-798 [PMID: 16116312]
58 **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]
59 **Giacaman RA**, Asrani AC, Ross KF, Herzberg MC. Cleavage of protease-activated receptors on an immortalized oral epithelial cell line by Porphyromonas gingivalis gingipains. *Microbiology* 2009; **155**: 3238-3246 [PMID: 19608609 DOI: 10.1099/mic.0.029132-0]
60 **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/S0002-9440(10)64466-5]
61 **Cuffe JE**, Bertog M, Velázquez-Rocha S, Dery O, Bunnett N, Korbmacher C. Basolateral PAR-2 receptors mediate KCl secretion and inhibition of Na+ absorption in the mouse distal colon. *J Physiol* 2002; **539**: 209-222 [PMID: 11850514]
62 **Kong W**, McConalogue K, Khitin LM, Hollenberg MD, Payan DG, Böhm SK, Bunnett NW. Luminal trypsin may regulate enterocytes through proteinase-activated receptor 2. *Proc Natl Acad Sci USA* 1997; **94**: 8884-8889 [PMID: 9238072]
63 **Holzhausen M**, Spolidorio LC, Ellen RP, Jobin MC, Steinhoff M, Andrade-Gordon P, Vergnolle N. Protease-activated receptor-2 activation: a major role in the pathogenesis of Porphyromonas gingivalis infection. *Am J Pathol* 2006; **168**: 1189-1199 [PMID: 16565494 DOI: 10.2353/ajpath.2006.050658]
64 **Vergnolle N**. Proteinase-activated receptor-2-activating peptides induce leukocyte rolling, adhesion, and extravasation in vivo. *J Immunol* 1999; **163**: 5064-5069 [PMID: 10528212]
65 **Vergnolle N**, Wallace JL, Bunnett NW, Hollenberg MD. Protease-activated receptors in inflammation, neuronal signaling and pain. *Trends Pharmacol Sci* 2001; **22**: 146-152 [PMID: 11239578]
66 **Hyun E**, Andrade-Gordon P, Steinhoff M, Vergnolle N. Protease-activated receptor-2 activation: a major actor in intestinal inflammation. *Gut* 2008; **57**: 1222-1229 [PMID: 18460552 DOI: 10.1136/gut.2008.150722]
67 **Lohman RJ**, Cotterell AJ, Barry GD, Liu L, Suen JY, Vesey DA, Fairlie DP. An antagonist of human protease activated receptor-2 attenuates PAR2 signaling, macrophage activation, mast cell degranulation, and collagen-induced arthritis in rats. *FASEB J* 2012; **26**: 2877-2887 [PMID: 22467762 DOI: 10.1096/fj.11-201004]
68 **Fiorucci S**, Mencarelli A, Palazzetti B, Distrutti E, Vergnolle N, Hollenberg MD, Wallace JL, Morelli A, Cirino G. Proteinase-activated receptor 2 is an anti-inflammatory signal for colonic lamina propria lymphocytes in a mouse model of colitis. *Proc Natl Acad Sci USA* 2001; **98**: 13936-13941 [PMID: 11717450 DOI: 10.1073/pnas.241377298]
69 **Nhu QM**, Shirey KA, Pennini ME, Stiltz J, Vogel SN. Proteinase-activated receptor 2 activation promotes an anti-inflammatory and alternatively activated phenotype in LPS-stimulated murine macrophages. *Innate Immun* 2012; **18**: 193-203 [PMID: 21239455 DOI: 10.1177/1753425910395044]
70 **Bustos D**, Negri G, De Paula JA, Di Carlo M, Yapur V, Facente A, De Paula A. Colonic proteinases: increased activity in patients with ulcerative colitis. *Medicina* (B Aires) 1998; **58**: 262-264 [PMID: 9713093]
71 **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]
72 **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]
73 **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]
74 **Lee JW**, Park JH, Park DI, Park JH, Kim HJ, Cho YK, Sohn CI, Jeon WK, Kim BI. Subjects with diarrhea-predominant IBS have increased rectal permeability responsive to tryptase. *Dig Dis Sci* 2010; **55**: 2922-2928 [PMID: 20087660 DOI: 10.1007/s10620-009-1094-8]
75 **Cottrell GS**, Amadesi S, Pikios S, Camerer E, Willardsen JA, Murphy BR, Caughey GH, Wolters PJ, Coughlin SR, Peterson A, Knecht W, Pothoulakis C, Bunnett NW, Grady EF. Protease-activated receptor 2, dipeptidyl peptidase I, and proteases mediate Clostridium difficile toxin A enteritis. *Gastroenterology* 2007; **132**: 2422-2437 [PMID: 17570216 DOI: 10.1053/j.gastro.2007.03.101]
76 **Hansen KK**, Sherman PM, Cellars L, Andrade-Gordon P, Pan Z, Baruch A, Wallace JL, Hollenberg MD, Vergnolle N. A major role for proteolytic activity and proteinase-activated receptor-2 in the pathogenesis of infectious colitis. *Proc Natl Acad Sci USA* 2005; **102**: 8363-8368 [PMID: 15919826 DOI: 10.1073/pnas.0409535102]
77 **Róka R**, Ait-Belgnaoui A, Salvador-Cartier C, Garcia-Villar R, Fioramonti J, Eutamène H, Bueno L. Dexamethasone prevents visceral hyperalgesia but not colonic permeability increase induced by luminal protease-activated receptor-2 agonist in rats. *Gut* 2007; **56**: 1072-1078 [PMID: 17309885 DOI: 10.1136/gut.2006.115352]
78 **Nakanishi-Matsui M**, Zheng YW, Sulciner DJ, Weiss EJ, Ludeman MJ, Coughlin SR. PAR3 is a cofactor for PAR4 activation by thrombin. *Nature* 2000; **404**: 609-613 [PMID: 10766244 DOI: 10.1038/35007085]
79 **McLaughlin JN**, Patterson MM, Malik AB. Protease-activated receptor-3 (PAR3) regulates PAR1 signaling by receptor dimerization. *Proc Natl Acad Sci USA* 2007; **104**: 5662-5667 [PMID: 17376866 DOI: 10.1073/pnas.0700763104]
80 **Mulè F**, Pizzuti R, Capparelli A, Vergnolle N. Evidence for the presence of functional protease activated receptor 4 (PAR4) in the rat colon. *Gut* 2004; **53**: 229-234 [PMID: 14724155]
81 **Sambrano GR**, Huang W, Faruqi T, Mahrus S, Craik C, Coughlin SR. Cathepsin G activates protease-activated receptor-4 in human platelets. *J Biol Chem* 2000; **275**: 6819-6823 [PMID: 10702240]
82 **Asfaha S**, Cenac N, Houle S, Altier C, Papez MD, Nguyen C, Steinhoff M, Chapman K, Zamponi GW, Vergnolle N. Protease-activated receptor-4: a novel mechanism of inflammatory pain modulation. *Br J Pharmacol* 2007; **150**: 176-185 [PMID: 17179954 DOI: 10.1038/sj.bjp.0706975]
83 **Vergnolle N**, Derian CK, D'Andrea MR, Steinhoff M, Andrade-Gordon P. Characterization of thrombin-induced leukocyte rolling and adherence: a potential proinflammatory role for proteinase-activated receptor-4. *J Immunol* 2002;

169: 1467-1473 [PMID: 12133973]
84 **Hollenberg MD**, Saifeddine M, Sandhu S, Houle S, Vergnolle N. Proteinase-activated receptor-4: evaluation of tethered ligand-derived peptides as probes for receptor function and as inflammatory agonists in vivo. *Br J Pharmacol* 2004; **143**: 443-454 [PMID: 15451771 DOI: 10.1038/sj.bjp.0705946]
85 **Dabek M**, Ferrier L, Annahazi A, Bézirard V, Polizzi A, Cartier C, Leveque M, Roka R, Wittmann T, Theodorou V, Bueno L. Intracolonic infusion of fecal supernatants from ulcerative colitis patients triggers altered permeability and inflammation in mice: role of cathepsin G and protease-activated receptor-4. *Inflamm Bowel Dis* 2011; **17**: 1409-1414 [PMID: 21560201 DOI: 10.1002/ibd.21454]
86 **Kim SC**, Tonkonogy SL, Albright CA, Tsang J, Balish EJ, Braun J, Huycke MM, Sartor RB. Variable phenotypes of enterocolitis in interleukin 10-deficient mice monoassociated with two different commensal bacteria. *Gastroenterology* 2005; **128**: 891-906 [PMID: 15825073]
87 **Mäkinen PL**, Clewell DB, An F, Mäkinen KK. Purification and substrate specificity of a strongly hydrophobic extracellular metalloendopeptidase ("gelatinase") from Streptococcus faecalis (strain 0G1-10). *J Biol Chem* 1989; **264**: 3325-3334 [PMID: 2536744]
88 **Mäkinen PL**, Mäkinen KK. The Enterococcus faecalis extracellular metalloendopeptidase (EC 3.4.24.30; coccolysin) inactivates human endothelin at bonds involving hydrophobic amino acid residues. *Biochem Biophys Res Commun* 1994; **200**: 981-985 [PMID: 8179636 DOI: 10.1006/bbrc.1994.1546]
89 **Park SY**, Kim KM, Lee JH, Seo SJ, Lee IH. Extracellular gelatinase of Enterococcus faecalis destroys a defense system in insect hemolymph and human serum. *Infect Immun* 2007; **75**: 1861-1869 [PMID: 17261598 DOI: 10.1128/IAI.01473-06]
90 **Park SY**, Shin YP, Kim CH, Park HJ, Seong YS, Kim BS, Seo SJ, Lee IH. Immune evasion of Enterococcus faecalis by an extracellular gelatinase that cleaves C3 and iC3b. *J Immunol* 2008; **181**: 6328-6336 [PMID: 18941224]
91 **Schmidtchen A**, Frick IM, Andersson E, Tapper H, Björck L. Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. *Mol Microbiol* 2002; **46**: 157-168 [PMID: 12366839]
92 **Waters CM**, Antiporta MH, Murray BE, Dunny GM. Role of the Enterococcus faecalis GelE protease in determination of cellular chain length, supernatant pheromone levels, and degradation of fibrin and misfolded surface proteins. *J Bacteriol* 2003; **185**: 3613-3623 [PMID: 12775699]
93 **Annaházi A**, Gecse K, Dabek M, Ait-Belgnaoui A, Rosztóczy A, Róka R, Molnár T, Theodorou V, Wittmann T, Bueno L, Eutamene H. Fecal proteases from diarrheic-IBS and ulcerative colitis patients exert opposite effect on visceral sensitivity in mice. *Pain* 2009; **144**: 209-217 [PMID: 19450926 DOI: 10.1016/j.pain.2009.04.017]
94 **Gecse K**, Róka R, Ferrier L, Leveque M, Eutamene H, Cartier C, Ait-Belgnaoui A, Rosztóczy A, Izbéki F, Fioramonti J, Wittmann T, Bueno L. Increased faecal serine protease activity in diarrhoeic IBS patients: a colonic lumenal factor impairing colonic permeability and sensitivity. *Gut* 2008; **57**: 591-599 [PMID: 18194983]
95 **Clavel T**, Haller D. Molecular interactions between bacteria, the epithelium, and the mucosal immune system in the intestinal tract: implications for chronic inflammation. *Curr Issues Intest Microbiol* 2007; **8**: 25-43 [PMID: 17542334]
96 **Imamura T**. The role of gingipains in the pathogenesis of periodontal disease. *J Periodontol* 2003; **74**: 111-118 [PMID: 12593605 DOI: 10.1902/jop.2003.74.1.111]
97 **Mikolajczyk-Pawlinska J**, Travis J, Potempa J. Modulation of interleukin-8 activity by gingipains from Porphyromonas gingivalis: implications for pathogenicity of periodontal disease. *FEBS Lett* 1998; **440**: 282-286 [PMID: 9872387]
98 **Edwards RJ**, Taylor GW, Ferguson M, Murray S, Rendell N, Wrigley A, Bai Z, Boyle J, Finney SJ, Jones A, Russell HH, Turner C, Cohen J, Faulkner L, Sriskandan S. Specific C-terminal cleavage and inactivation of interleukin-8 by invasive disease isolates of Streptococcus pyogenes. *J Infect Dis* 2005; **192**: 783-790 [PMID: 16088827 DOI: 10.1086/432485]
99 **Zinkernagel AS**, Timmer AM, Pence MA, Locke JB, Buchanan JT, Turner CE, Mishalian I, Sriskandan S, Hanski E, Nizet V. The IL-8 protease SpyCEP/ScpC of group A Streptococcus promotes resistance to neutrophil killing. *Cell Host Microbe* 2008; **4**: 170-178 [PMID: 18692776 DOI: 10.1016/j.chom.2008.07.002]
100 **Chiang-Ni C**, Zheng PX, Ho YR, Wu HM, Chuang WJ, Lin YS, Lin MT, Liu CC, Wu JJ. emm1/sequence type 28 strains of group A streptococci that express covR at early stationary phase are associated with increased growth and earlier SpeB secretion. *J Clin Microbiol* 2009; **47**: 3161-3169 [PMID: 19710271 DOI: 10.1128/JCM.00202-09]
101 **Smagur J**, Guzik K, Magiera L, Bzowska M, Gruca M, Thøgersen IB, Enghild JJ, Potempa J. A new pathway of staphylococcal pathogenesis: apoptosis-like death induced by Staphopain B in human neutrophils and monocytes. *J Innate Immun* 2009; **1**: 98-108 [PMID: 20375568 DOI: 10.1159/000181014]
102 **Manichanh C**, Borruel N, Casellas F, Guarner F. The gut microbiota in IBD. *Nat Rev Gastroenterol Hepatol* 2012; **9**: 599-608 [PMID: 22907164 DOI: 10.1038/nrgastro.2012.152]
103 **Gionchetti P**, Rizzello F, Helwig U, Venturi A, Lammers KM, Brigidi P, Vitali B, Poggioli G, Miglioli M, Campieri M. Prophylaxis of pouchitis onset with probiotic therapy: a double-blind, placebo-controlled trial. *Gastroenterology* 2003; **124**: 1202-1209 [PMID: 12730861]
104 **Shen B**. Acute and chronic pouchitis--pathogenesis, diagnosis and treatment. *Nat Rev Gastroenterol Hepatol* 2012; **9**: 323-333 [PMID: 22508158 DOI: 10.1038/nrgastro.2012.58]
105 **Ohland CL**, Macnaughton WK. Probiotic bacteria and intestinal epithelial barrier function. *Am J Physiol Gastrointest Liver Physiol* 2010; **298**: G807-G819 [PMID: 20299599 DOI: 10.1152/ajpgi.00243.2009]
106 **Pimentel M**, Lembo A, Chey WD, Zakko S, Ringel Y, Yu J, Mareya SM, Shaw AL, Bortey E, Forbes WP. Rifaximin therapy for patients with irritable bowel syndrome without constipation. *N Engl J Med* 2011; **364**: 22-32 [PMID: 21208106]
107 **Madsen K**, Cornish A, Soper P, McKaigney C, Jijon H, Yachimec C, Doyle J, Jewell L, De Simone C. Probiotic bacteria enhance murine and human intestinal epithelial barrier function. *Gastroenterology* 2001; **121**: 580-591 [PMID: 11522742]
108 **Mowat C**, Cole A, Windsor A, Ahmad T, Arnott I, Driscoll R, Mitton S, Orchard T, Rutter M, Younge L, Lees C, Ho GT, Satsangi J, Bloom S. Guidelines for the management of inflammatory bowel disease in adults. *Gut* 2011; **60**: 571-607 [PMID: 21464096 DOI: 10.1136/gut.2010.224154]
109 **Löwer M**, Geppert T, Schneider P, Hoy B, Wessler S, Schneider G. Inhibitors of Helicobacter pylori protease HtrA found by 'virtual ligand' screening combat bacterial invasion of epithelia. *PLoS One* 2011; **6**: e17986 [PMID: 21483848 DOI: 10.1371/journal.pone.0017986]
110 **Hoy B**, Geppert T, Boehm M, Reisen F, Plattner P, Gadermaier G, Sewald N, Ferreira F, Briza P, Schneider G, Backert S, Wessler S. Distinct roles of secreted HtrA proteases from gram-negative pathogens in cleaving the junctional protein and tumor suppressor E-cadherin. *J Biol Chem* 2012; **287**: 10115-10120 [PMID: 22337879 DOI: 10.1074/jbc.C111.333419]
111 **Ivanov D**, Emonet C, Foata F, Affolter M, Delley M, Fisseha M, Blum-Sperisen S, Kochhar S, Arigoni F. A serpin from the gut bacterium Bifidobacterium longum inhibits eukaryotic elastase-like serine proteases. *J Biol Chem* 2006; **281**: 17246-17252 [PMID: 16627467 DOI: 10.1074/jbc.M601678200]
112 **Alvarez-Martin P**, O'Connell Motherway M, Turroni F,

Foroni E, Ventura M, van Sinderen D. A two-component regulatory system controls autoregulated serpin expression in Bifidobacterium breve UCC2003. *Appl Environ Microbiol* 2012; **78**: 7032-7041 [PMID: 22843530 DOI: 10.1128/AEM.01776-12]

113 **Castagliuolo I**, Riegler MF, Valenick L, LaMont JT, Pothoulakis C. Saccharomyces boulardii protease inhibits the effects of Clostridium difficile toxins A and B in human colonic mucosa. *Infect Immun* 1999; **67**: 302-307 [PMID: 9864230]

114 **Covic L**, Misra M, Badar J, Singh C, Kuliopulos A. Pepducin-based intervention of thrombin-receptor signaling and systemic platelet activation. *Nat Med* 2002; **8**: 1161-1165 [PMID: 12357249 DOI: 10.1038/nm760]

115 **Dimond P**, Carlson K, Bouvier M, Gerard C, Xu L, Covic L, Agarwal A, Ernst OP, Janz JM, Schwartz TW, Gardella TJ, Milligan G, Kuliopulos A, Sakmar TP, Hunt SW. G protein-coupled receptor modulation with pepducins: moving closer to the clinic. *Ann N Y Acad Sci* 2011; **1226**: 34-49 [PMID: 21615752 DOI: 10.1111/j.1749-6632.2011.06039.x]

116 **Cisowski J**, O'Callaghan K, Kuliopulos A, Yang J, Nguyen N, Deng Q, Yang E, Fogel M, Tressel S, Foley C, Agarwal A, Hunt SW, McMurry T, Brinckerhoff L, Covic L. Targeting protease-activated receptor-1 with cell-penetrating pepducins in lung cancer. *Am J Pathol* 2011; **179**: 513-523 [PMID: 21703428 DOI: 10.1016/j.ajpath.2011.03.025]

117 **Michael ES**, Kuliopulos A, Covic L, Steer ML, Perides G. Pharmacological inhibition of PAR2 with the pepducin P2pal-18S protects mice against acute experimental biliary pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 2013; **304**: G516-G526 [PMID: 23275617 DOI: 10.1152/ajpgi.00296.2012]

118 **Sevigny LM**, Zhang P, Bohm A, Lazarides K, Perides G, Covic L, Kuliopulos A. Interdicting protease-activated receptor-2-driven inflammation with cell-penetrating pepducins. *Proc Natl Acad Sci USA* 2011; **108**: 8491-8496 [PMID: 21536878 DOI: 10.1073/pnas.1017091108]

119 **Naglik J**, Albrecht A, Bader O, Hube B. Candida albicans proteinases and host/pathogen interactions. *Cell Microbiol* 2004; **6**: 915-926 [PMID: 15339267 DOI: 10.1111/j.1462-5822.2004.00439.x]

120 **Aly KA**, Beebe ET, Chan CH, Goren MA, Sepúlveda C, Makino S, Fox BG, Forest KT. Cell-free production of integral membrane aspartic acid proteases reveals zinc-dependent methyltransferase activity of the Pseudomonas aeruginosa prepilin peptidase PilD. *Microbiologyopen* 2013; **2**: 94-104 [PMID: 23255525 DOI: 10.1002/mbo3.51]

121 **Bardy SL**, Jarrell KF. Cleavage of preflagellins by an aspartic acid signal peptidase is essential for flagellation in the archaeon Methanococcus voltae. *Mol Microbiol* 2003; **50**: 1339-1347 [PMID: 14622420]

122 **Marraffini LA**, Dedent AC, Schneewind O. Sortases and the art of anchoring proteins to the envelopes of gram-positive bacteria. *Microbiol Mol Biol Rev* 2006; **70**: 192-221 [PMID: 16524923 DOI: 10.1128/MMBR.70.1.192-221.2006]

123 **Massimi I**, Park E, Rice K, Muller-Esterl W, Sauder D, McGavin MJ. Identification of a novel maturation mechanism and restricted substrate specificity for the SspB cysteine protease of Staphylococcus aureus. *J Biol Chem* 2002; **277**: 41770-41777 [PMID: 12207024 DOI: 10.1074/jbc.M207162200]

124 **Thornton RF**, Kagawa TF, O'Toole PW, Cooney JC. The dissemination of C10 cysteine protease genes in Bacteroides fragilis by mobile genetic elements. *BMC Microbiol* 2010; **10**: 122 [PMID: 20416045 DOI: 10.1186/1471-2180-10-122]

125 **Hoy B**, Löwer M, Weydig C, Carra G, Tegtmeyer N, Geppert T, Schröder P, Sewald N, Backert S, Schneider G, Wessler S. Helicobacter pylori HtrA is a new secreted virulence factor that cleaves E-cadherin to disrupt intercellular adhesion. *EMBO Rep* 2010; **11**: 798-804 [PMID: 20814423 DOI: 10.1038/embor.2010.114]

126 **Koide Y**, Nakamura A, Uozumi T, Beppu T. Cloning and sequencing of the major intracellular serine protease gene of Bacillus subtilis. *J Bacteriol* 1986; **167**: 110-116 [PMID: 3087947]

P- Reviewers: da Silva Figueredo C, Jiang GL, Maltz C, Tsai HH
S- Editor: Zhai HH **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.v19.i43.7544

World J Gastroenterol 2013 November 21; 19(43): 7544-7551
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Beyond white light endoscopy: The role of optical biopsy in inflammatory bowel disease

Julia Liu, Aldona Dlugosz, Helmut Neumann

Julia Liu, Division of Gastroenterology, the Centre of Excellence for Gastrointestinal Inflammation and Immunity Research, University of Alberta, Alberta T6G 2X8, Canada
Aldona Dlugosz, Division of Gastroenterology and Hepatology, Department of Medicine, Karolinska University Hospital, Karolinska Institutet, SE-141 86 Stockholm, Sweden
Helmut Neumann, Department of Medicine 1, Interdisciplinary Endoscopy, University of Erlangen-Nuremberg, 91054 Erlangen, Germany
Author contributions: Liu J wrote the abstract, introduction, clinical applications for disease relapse and research applications sections; Dlugosz A wrote the section on dysplasia detection in inflammatory bowel disease (IBD); Neumann H wrote the sections on the technical aspects of optical biopsy and assessment of inflammation in IBD.
Supported by Dr. Julia Liu is a recipient of the Canadian Institute of Health Research New Investigator Salary Award, and funding support from the Canadian Association of Gastroenterology and Crohn's and Colitis Foundation of Canada; Dr. Aldona Dlugosz is a recipient of the Postdoctoral Fellowship from Stockholm County Council and funding support from the Karolinska Institute Funds; Dr. Helmut Neumann is a recipient of the 2013 American Society for Gastrointestinal Endoscopy Cook Medical Don Wilson Award, this work was done during the award period
Correspondence to: Julia Liu, MD, MSc, Division of Gastroenterology, the Centre of Excellence for Gastrointestinal Inflammation and Immunity Research, University of Alberta, 1-10 Zeidler Ledcor Centre, Alberta T6G 2X8, Canada. julia.liu@ualberta.ca
Telephone: +1-780-4927062 Fax: +1-780-4927115
Received: August 7, 2013 Revised: October 8, 2013
Accepted: October 17, 2013
Published online: November 21, 2013

Abstract

In this review, we will discuss the use of two optical biopsy modalities in inflammatory bowel disease (IBD). The two techniques reviewed here are confocal laser endomicroscopy and endocytoscopy. We will describe the technical performance of the procedure, discuss the clinical indications for optical biopsy in IBD, and highlight active research areas with respect to the pathogenesis of IBD. Clinical indications for optical biopsies in IBD include assessment of mucosal inflammation, dysplasia detection and evaluation of cell shedding for disease relapse. Research application in the area of barrier dysfunction will also be discussed.

Key words: Optical biopsy; Confocal endomicroscopy; Endocytoscopy; Dysplasia; Mucosal inflammation; Disease relapse; Mucosal healing; Barrier function

Core tip: This is a review of the latest advances in the applications of optical biopsy (either with confocal laser endomicroscopy of endocytoscopy) in inflammatory bowel disease. Clinical indications including assessment of mucosal inflammation, detection of dysplasia and predictors for disease relapse are discussed in detail. Novel research use of optical biopsy for functional mucosal assessment is also discussed.

Liu J, Dlugosz A, Neumann H. Beyond white light endoscopy: The role of optical biopsy in inflammatory bowel disease. *World J Gastroenterol* 2013; 19(43): 7544-7551 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7544.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7544

INTRODUCTION

In the past decade, several advanced endoscopic imaging technologies that enable clinicians to examine the luminal gastrointestinal tract at a microscopic level were introduced. These techniques are called optical biopsies, as they are real-time histologic biopsies of the tissue. These include confocal laser endomicroscopy (CLE) and

endocytoscopy (EC). The CLE and EC systems come as either probe-based (pCLE and pEC) or endoscope-based (iCLE and iEC). In this review, we will describe the technical performance of the procedure, and discuss the clinical indications for optical biopsy in relation to inflammatory bowel diseases (IBD). We will also highlight some active research applications for optical biopsy in our understanding of the pathogenesis of IBD.

TECHNICAL ASPECTS OF OPTICAL BIOPSY

CLE was introduced in 2003 and allows *in vivo* microscopic imaging of cellular and subcellular structures at approximately 1000-fold magnification[1]. The technique is based on tissue illumination with a low power laser after application of fluorescence agents, which can either be applied systemically (*i.e.*, fluorescein sodium) or topically (*e.g.*, acriflavine hydrochloride, cresyl violet). The laser light is reflected from the tissue and then refocused onto the detection system by the same lens, so that only returning light refocused through the pinhole is detected. Therefore, this process decreases the effect of scattered light resulting in the construction of two-dimensional grey-scale images.

Currently, two CE certified and Food and Drug Administration approved CLE-devices are available[1]. One is integrated into the distal tip of a standard, high-resolution endoscope (iCLE, Pentax, Tokyo, Japan). The other one is probe based, capable of passage through the accessory channel of any standard endoscope (pCLE, Cellvizio, Mauna Kea Technologies, Paris, France). Both systems use an incident 488 nm wavelength laser (blue laser light) enabling the detection of fluorescence between 205 nm and 585 nm wavelengths.

The iCLE-system collects images at a manually adjustable scan rate of 1.6 frames per second with a resolution of 1024 × 512 pixels, or at 0.8 frames per second with a resolution of 1024 × 1024 pixels with dynamically adjustable depth of scanning ranging from 0 to 250 μm. The examiner can manually adjust the laser power between 0 and 1000 μW and the optical slice thickness is 7 μm, with lateral and axial resolution of 0.7 μm and a confocal image field of view of 475 × 475 μm. The pCLE-system is a stand-alone confocal probe which is advanced through the working channel of any endoscope and could thereby also being used with high-definition video endoscopes in combination with dye-less chromoendoscopy (*e.g.*, Narrow Band Imaging, Fuji Intelligent Chromo Endoscopy, i-scan) as red-flag techniques.

pCLE-systems are available for different indications throughout the entire gastrointestinal tract and use a fixed laser power and a fixed image plane depth which is dependent on the probe type used. Confocal images are streamed at a frame rate of 12 frames per second. CLE in IBD is mostly being performed by using the ColoFlex Ultra-High Definition probe which requires a 2.8 mm working channel to be advanced through the scope. Lateral resolution is 1 μm and field of view is 240 μm with an imaging plane depth of 65 μm. In addition, a special computer algorithm ("mosaicing") allows reconstruction of single video frames with an increased field of view (4 mm × 2 mm). Costs for single probes vary and are approximately 100-200 Euro per procedure. Like any other endoscopic technique CLE requires special training in performing the procedure and interpretation of images. Therefore, especially at the beginning, extensive close collaboration with an expert histopathologist is strictly recommended. In addition, when starting with CLE optical biopsies should always be compared with physical biopsies. After an appropriate learning phase, CLE interpretation shows high inter- and intraobserver variabilities as compared to standard histology. Both CLE-systems have unique advantages. Advantages of the integrated system are its higher resolution and the possibility to alter the laser power and imaging plane depth. Advantages of the probe-based system include the possibility of an *ad hoc* usage and a greater versatility of the pCLE probes, which can be used with nearly any endoscopes.

In contrast to CLE, endocytoscopy (EC; Olympus, Tokyo, Japan) is based on the principle of contact light microscopy[2]. EC-systems are either integrated into the distal tip of a standard endoscope (iEC) or probe-based (pEC). Through-the-scope pEC-systems require a working channel of at least 3.2 mm. Similar to contact light microscopy, EC requires thorough mucolysis with N-acetyl cysteine followed by staining of the mucosa with absorptive staining agents, like methylene blue, toluidine blue, or cresyl violet. In fact, a combination of different dye agents is often used to acquire optimal tissue contrast[3]. After an appropriate time of exposure to the dye (approximately 60 s), repeat washing of the mucosa is necessary to remove the excess contrast dye before endocytoscopic imaging. Repeat staining is mostly necessary while using absorptive contrast agents. Depending on the system used (iEC or pEC), EC visualizes architectural details (*e.g.*, epithelial structure), cellular features (*e.g.*, size and arrangement of cells), and vascular pattern morphology (*e.g.*, size and tortuosity) at a magnification of up to 1390-fold[2,3]. Representative image of normal colon mucosa is shown in Figure 1.

CLINICAL APPLICATIONS: ASSESSMENT OF INFLAMMATION IN IBD

Endomicroscopy

Accurate assessment of mucosal inflammation in patients with IBD is of crucial importance as mucosal healing has emerged as an important treatment goal and appears to be of paramount importance for optimized medical therapy[4]. Various studies and one recent systematic review has shown that mucosal healing as assessed by endoscopy is predictive of reduced disease activity, a decreased need for active treatment, reduced rates of hospitalizations/surgical resections, and is associated with sustained clinical remission[4-7]. While standard white-light endoscopy is

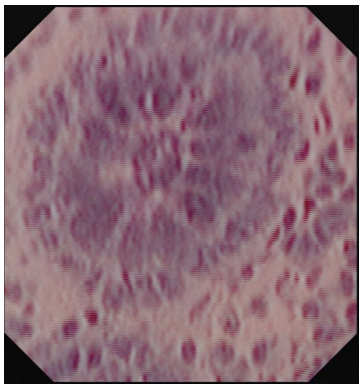


Figure 1 Endocytoscopic image at magnification × 1390 showing one single colonic crypt. Goblet and epithelial cells are clearly evident.

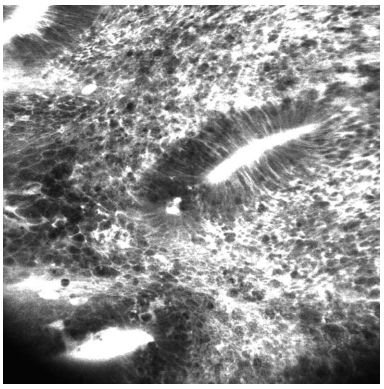


Figure 2 Endoscope-based confocal laser endomicroscopy of Crohn's disease shows remarkable cell infiltration, disturbed colonic architecture and leakage indicating severe inflammation.

likely an insensitive test for assessment of mucosal healing, being false negative in up to fifty percent of patients, there is an urgent need for new endoscopic imaging techniques allowing assessment of microscopic inflammation even in case of macroscopic non-inflamed mucosa[8,9]. In this context CLE was proven to be efficient for real-time *in vivo* assessment of mucosal inflammation by requiring only a short learning curve[10].

One early study investigated the features of CLE in inflamed and non-inflamed rectal mucosa and compared these results to standard histology[11]. On CLE, colonic crypts of normal colonic mucosa were small, round and regularly arranged, and the crypt lumens of the colonic glands were small and round. In contrast colonic crypts in non-active ulcerative colitis were small, round and slightly irregular in arrangement and the crypt lumens of the colonic glands were small and round. Inflammatory cells and capillaries were visible in the lamina propria. The colonic crypts in active ulcerative colitis were large, variously shaped and irregular in arrangement and in addition numerous inflammatory cells and capillaries were visible in the lamina propria. Li *et al*[12] confirmed these early results in a study including 73 consecutive patients. CLE-assessment of crypt architecture and fluorescein leakage showed good correlation with the corresponding histology results. Of note, more than half of the patients with normal mucosa seen on conventional white-light endoscopy revealed acute inflammation on histology, whereas no patients with normal mucosa or with chronic inflammation seen on CLE showed acute inflammation on histology. Therefore, CLE appears to be a sensitive tool for real-time assessment of inflammatory activity in patients with ulcerative colitis.

For Crohn's disease, our group evaluated in a case-control study whether CLE is feasible for *in vivo* diagnosis of Crohn's disease associated histological changes[13]. It was shown that a significantly higher proportion of patients with active Crohn's disease had increased colonic crypt tortuosity, enlarged crypt lumen, microerosions, augmented vascularization, and increased cellular infiltrates within the lamina propria. In quiescent Crohn's disease, a significant increase in crypt and goblet cell number was detected compared with controls. Based on these findings, the Crohn's Disease Endomicroscopic Activity Score was proposed, allowing the assessment of Crohn's disease activity *in vivo,* even in macroscopically non-inflamed mucosa. Representative iCLE and pCLE images of Crohn's disease are shown in Figures 2 and 3. Taken these and the above mentioned results into account, CLE is reliable for real time *in vivo* assessment of microscopic inflammation in patients with IBD and macroscopically non-inflamed mucosa.

As most commonly used drugs for treatment of IBD are systemically bioavailable, they cover a potential risk of severe side effects. Therefore, a targeted, individualized approach to inflamed areas of the intestine with specific drugs is highly desirable. In this context, one recent study evaluated the potential of nanoparticle and microparticle uptake into the rectal mucosa of human IBD patients[14]. CLE was performed two hours after rectal application of fluorescent-labeled placebo nanoparticles and microparticles to 33 patients with IBD and healthy controls in order to visualize the particles in inflamed mucosal areas. A significantly enhanced accumulation of microparticles was observed in ulcerated areas, whereas nanoparticles were only visible in trace amount on mucosal surfaces of normal patients. Therefore, nanoparticles may enable local drug delivery to intestinal lesions in humans, thereby minimizing the risk of unintended translocation into the blood system. Very recently, our group created a fluorescent labeled antibody for molecular membrane-bound tumor necrosis factor (mTNF) imaging in Crohn's disease patients[15]. Topical antibody administration led to detection of intestinal mTNF positive immune cells during CLE. Interestingly, patients with high amounts of mTNF positive cells showed significantly higher short-term response rates at week 12 (92%) upon subsequent anti-TNF therapy as compared to patients with low amounts of mTNF positive cells (15%). This clinical response in the former patients was sustained over a follow-up period of one year and associated with mucosal healing on follow-up endoscopy. These results are promising and offer the exciting potential for individualized therapy for IBD patients using molecular imaging with fluorescent labeled antibodies.

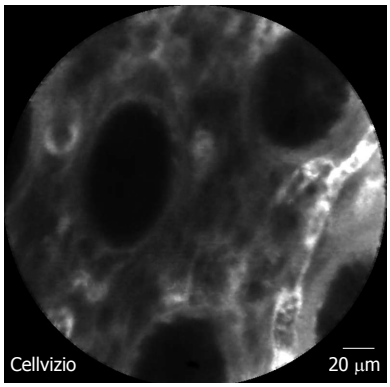


Figure 3 Probe-based confocal laser endomicroscopy in patient with Crohn's disease without histologic activity. Colonic crypts are regularly arranged with normal shape and size. Micro-vessels within the lamina propria are easily visible. Bar = 20 μm.

Endocytoscopy

Only limited data are currently available on the potential of EC in patients with IBD. One recent article correlated the efficacy between endocytoscopy and conventional histopathology for assessment of microscopic features in patients with ulcerative colitis[16]. Fifty-five patients were included and mucosal patterns were evaluated by using EC with × 450 magnification. Based on EC-findings a scoring system was developed that showed a strong correlation with Matts' histopathological grades. In addition, there was a strong correlation between the conventional Matts' endoscopic grade and Matts' histopathological grade. Furthermore, the newly developed EC-score showed high reproducibility among investigators with a κ value of 0.79. Another recent study determined the reliability of EC for the discrimination of mucosal inflammatory cells and intestinal inflammatory disease activity in patients with IBD[17]. In total, 40 patients were included and EC was reliable to distinguish single inflammatory cells, including neutrophilic, basophilic, and eosinophilic granulocytes and lymphocytes. Sensitivity and specificity ranges among different cell types between 60% and 89% and 90% and 95%, respectively. Interobserver agreement among investigators was substantial whereas intraobserver agreement was almost perfect. Moreover, concordance between endocytoscopy and histopathology for grading of intestinal disease activity was 100%.

In conclusion, EC holds significant potential in identifying early signs of mucosal inflammation in real-time by identifying single mucosal inflammatory cells in conjunction with architectural details. Large, prospective multicenter trials evaluating EC for prediction of disease course in IBD are thus highly warranted and anticipated.

CLINICAL APPLICATIONS: DYSPLASIA DETECTION IN IBD

Patients with IBD are at an increased risk for the development of dysplasia (also known as intraepithelial neoplasia) and colitis associated cancer (CAC). The risk of developing CAC is comparable for Crohn's colitis and ulcerative colitis patients. In both cases the risk is increased with: duration of colitis, early age of IBD onset, extent of colonic involvement, severity of inflammation, family history of colorectal cancer and particularly the presence of primary sclerosing cholangitis[18]. The cumulative risk for developing CAC in primary sclerosing cholangitis associated IBD (PSC-IBD) patients is 33% at 20 years and 40% at 30 years compared to 8% and 18% in IBD without concomitant liver disease[19]. Although IBDs contribute only 1%-2% to all cases of colorectal cancer, the cancer-related mortality rate in IBD patients is approximately 15%[20,21].

Colonoscopy with random biopsies, which is the gold standard for CAC screening in long standing IBD, can significantly reduce CAC-related mortality[22] and IBD patients undergoing surveillance colonoscopy had detection of neoplasia at an earlier stage, resulting in a better corresponding prognosis[23]. The principal limitation with this modality is that neoplasia may not be appreciated in up to a third of colonoscopies[24,25]. Taking four-quadrant biopsies is time-consuming and has only moderate sensitivity for neoplasia detection. The efficacy of surveillance for detection of early malignancies could also be questioned from the standpoint of cost and adherence. A population-based analysis of the cost and practice of colonic surveillance of patients with PSC-IBD in Alberta, Canada revealed that only 1/3 of the colonoscopies expected were actually performed, but despite suboptimal surveillance, the incidence of colorectal neoplasia was high. The study also found that the cost of finding one additional case of dysplasia was substantial[26]. Moreover, dysplasia in IBD can be found at distant sites from the cancer itself or before the cancer develops and is difficult to recognize on colonoscopy, as it often arises from flat, normal-appearing mucosa[20]. Dysplasia can also occur within or near plaque-like lesions or raised polypoid masses, defined as dysplasia-associated lesion or mass (DALM).

Targeted biopsies are an attractive alternative to random biopsies to increase the yield of dysplasia detection. CLE allows evaluation of suspicious lesions at the subcellular level with great detail prior to performing targeted biopsies, thus facilitating earlier diagnosis of CAC. While CLE only covers a limited field of view within the mucosa, pan-CLE of the whole colon is not feasible. Therefore, macroscopic visualization of suspected areas with chromoendoscopy (red flag technique) is useful before performing targeted endomicroscopy[27,28]. Prospective evaluation of CLE with concurrent chromoendoscopy predicted neoplasia in a randomized controlled trial of ulcerative colitis patients (n = 153) with great accuracy (98%), sensitivity (95%) and specificity (98%). By using methylene-blue aided chromoendoscopy with CLE, the diagnostic yield of neoplasia was increased by 4.75-fold compared with conventional colonoscopy with random biopsies (P = 0.005), though 50% fewer biopsy specimens were required[29]. Hurlstone *et al*[30] demonstrated

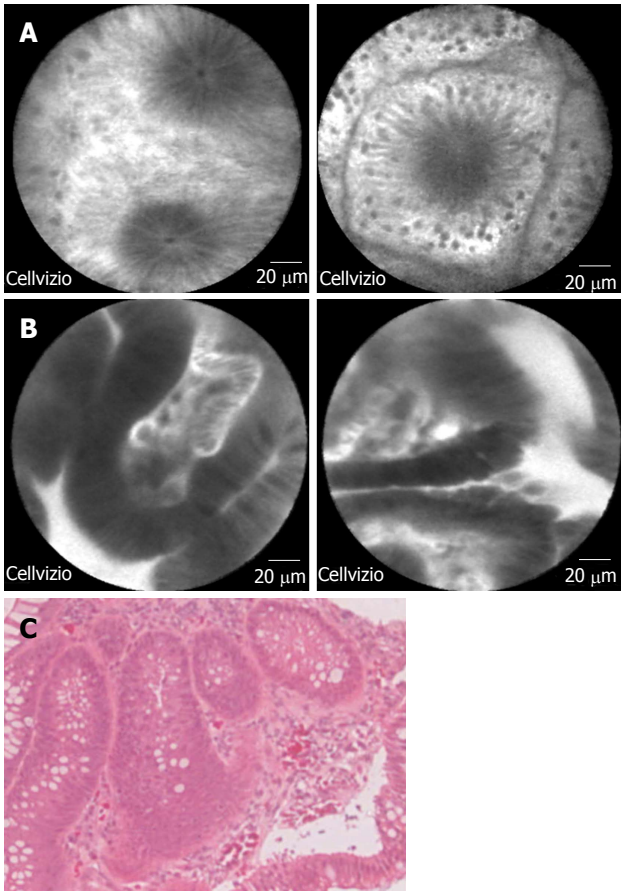


Figure 4 Probe-based confocal laser endomicroscopy images of (A) normal colonic mucosa; B: Adenomatous polyp with low grade dysplasia. Bar = 20 mm; and C: The corresponding histologic image for the adenomatous polyp with dysplasia (magnification × 40).

remarkable results in a smaller ulcerative colitis cohort (n = 36), where CLE enabled differentiation of DALM and adenoma-like mass with 97% accuracy and an excellent agreement between endomicroscopy and histopathological diagnosis was found (P = 0.91). CLE based, targeted biopsies increased the diagnostic yield of intraepithelial neoplasia by 2.5-fold compared with chromoendoscopy-guided biopsies alone. One recent pilot study of pCLE during colonoscopic surveillance of patients with long-standing ulcerative colitis (n = 22) demonstrated that the method is feasible with reasonable diagnostic accuracy[31]. A recent meta-analysis of 15 studies of confocal endomicroscopy for dysplasia detection[32] showed CLE could distinguish neoplasms from non-neoplasms in IBD patients for surveillance with a sensitivity of 0.83 (95%CI: 0.70-0.92) and specificity of 0.90 (95%CI: 0.87-0.93). Representative pCLE images of adenomatous polyp with low grade dysplasia and corresponding histologic images are shown in Figure 4.

CLE seems to be a particularly promising method for dysplasia detection in PSC-IBD patients. PSC-IBD is recently suggested to represent a specific IBD phenotype characterized by extensive colitis, low inflammatory activity, right-sided colonic inflammation and a high risk of CAC. Jørgensen *et al*[33] showed a difference between the macroscopic and microscopic picture in PSC-IBD: in general the inflammatory activity in these patients was low and was not always visible endoscopically, though it could be seen histologically. Since proximal cancers are more common in PSC-IBD patients[34,35] and inflammatory activity is not always visible endoscopically the use of CLE may increase surveillance efficiency particularly in the right colon. One ongoing study evaluates the efficacy of pCLE as a complementary tool to high definition white-light endoscopy (HD-WLE) for the detection of colonic dysplasia in patients with PSC-IBD. Preliminary results (n = 25) showed excellent accuracy (99%), sensitivity (93%) and specificity (100%) of pCLE in dysplasia detection that was superior to HD-WLE alone[36]. Low-grade intraepithelial neoplasia was found in 20% of patients and 60% of confirmed dysplastic lesions were localized in the right colon. These preliminary results suggest that careful CLE examination of at least the right colon in PSC-IBD patients may be warranted.

CLINICAL APPLICATIONS: ASSESSMENT OF CELL EXTRUSION AND BARRIER FUNCTION FOR DISEASE RELAPSE

Mucosal healing has emerged as the most important endoscopic predictor of disease relapse in IBD patients. In the pre-biologic era, mucosal healing was associated with lower rate of relapse in ulcerative colitis but not Crohn's disease patients[6]. In the biologic era, mucosal healing is predictive of clinical and endoscopic remission for both Crohn's[7,37] and ulcerative colitis patients[38,39]. The use of optical biopsy in the small bowel in IBD patients have been studied by several groups[1,40,41]. The principal finding on CLE that appears to predict disease relapse is epithelial cell extrusion and associated barrier dysfunction[42,43]. Using CLE, Kiesslich *et al*[40] first reported unambiguous identification of epithelial gaps or extrusion zones in the intestine of patients. Epithelial cell extrusion occurs as part of a normal physiological renewal process of the intestine. Therefore, qualitative description of the presence or absence of epithelial gaps is not sufficient to discern a diseased from a healthy state. We have therefore developed a quantitative measure called epithelial gap density, defined as the total number of epithelial gaps counted normalized to the total number of epithelial cells counted on pCLE images[41]. Gap density was validated against conventional multi-photon confocal microscopy and conventional white-light microscopy[44]. The gap density was found to be increased in IBD patient[45]. More recently, increased gap density and certain types of epithelial gaps were found to be predictive of aggressive disease[43] and relapse in IBD patients[42]. Thus, qualitative and quantitative studies of epithelial gaps in the small intestine may have a role in defining new management algorithms for IBD patients.

The clinical applications of CLE (both pCLE and

iCLE) in the small intestine of IBD patients have been largely in the evaluation of the terminal ileum[40,42,43,45]. The terminal ileum is the preferred site of intestinal evaluation in IBD for a number of reasons: (1) it is within the reach of a standard colonoscope; (2) it is an active site of immunologic activity; (3) it is often the first site of disease in Crohn's patients; and (4) it is usually possible to find endoscopically normal appearing areas, even in patients with active Crohn's ileitis. We have devised a quantitative determination of the density of epithelial gaps or extrusion zone called the epithelial gap density in the terminal ileum[41,45]. The epithelial gap density derived using CLE could be validated against multi-photon confocal microscopy and light microscopy[46]. Increased epithelial gap density could identify patients at high risk for major events such as hospitalization or surgery in follow up[43]. In another recent study, certain types of epithelial gaps were found to be predictive of disease relapse in IBD patients[42]. The current evidence seems to suggest that quantification and evaluation of extrusion zones in the terminal ileum of IBD patients have predictive values for disease relapse. However, these are single-centered studies, and there are no multicenter trials to determine the role of CLE in the management of IBD patients.

RESEARCH APPLICATIONS: FUNCTIONAL MUCOSAL ASSESSMENT IN IBD

The intestinal epithelium functions as a selective barrier to the luminal contents[47,48]. The epithelium separates the immune and neural networks of the human intestine from the intestinal microbiota, which comprises an estimated 10^{13} to 10^{14} microorganisms[49]. Compromised epithelial barrier expose the subepithelial immune system to resident microbes which induces secretion of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α[50], which in turn induces shedding of epithelial cells from the intestine which then further contributes to barrier dysfunction and promotes inflammation[51]. Other proinflammatory cytokines, including interleukin (IL)-1β, IL-6, IL-18 and TNF-α have been reported to be increased in active IBD and correlate with endoscopic severity of inflammation[52,53].

Until advances of optical biopsy, either *via* CLE or EC, it was not possible to investigate the interactions between the intestinal epithelium and resident microbiota *in vivo*. Histologic evaluation of the intestinal mucosa is limited by fixation and processing artefacts, including contamination with luminal microbes. CLE was used to identify intramucoal bacteria, and IBD patients were found to have significantly higher distribution of involvement with intramucosal bacteria in the colon and terminal ileum[54]. CLE was recently used for molecular imaging to identify single bacteria species and *in vivo* diagnosis of bacteria associated colitis[55].

Barrier dysfunction is another area of research interest for the past few years. Restoration of normal epithelial barrier function which prevent the translocation of commensal bacteria is the structural basis of mucosal healing[4]. Intestinal permeability function as assessed with disaccharide solutions[56,57] has not been widely adopted for clinical use. Optical biopsy permits assessment of mucosal barrier function in the appropriate structural context. Studies have shown increased epithelial cell shedding may contribute to barrier dysfunction in the intestine[42,58]. The clinical relevance of cell shedding and barrier dysfunction is reflected in the ability of these measures to predict disease relapse and major events in follow up[42,43].

CONCLUSION

Optical biopsy of the intestine in IBD have been used for a variety of clinical and research indications, and appears to hold significant promise to improve the diagnosis and management of IBD patients. Future large, multicentered studies are needed to validate these early study findings to facilitate clinical adaptation of this group of new advanced imaging technique.

REFERENCES

1 **Neumann H**, Kiesslich R, Wallace MB, Neurath MF. Confocal laser endomicroscopy: technical advances and clinical applications. *Gastroenterology* 2010; **139**: 388-392, 392.e1-e2 [PMID: 20561523]

2 **Neumann H**, Fuchs FS, Vieth M, Atreya R, Siebler J, Kiesslich R, Neurath MF. Review article: in vivo imaging by endocytoscopy. *Aliment Pharmacol Ther* 2011; **33**: 1183-1193 [PMID: 21457290 DOI: 10.1111/j.1365-2036.2011.04647.x]

3 **Minami H**, Inoue H, Yokoyama A, Ikeda H, Satodate H, Hamatani S, Haji A, Kudo S. Recent advancement of observing living cells in the esophagus using CM double staining: endocytoscopic atypia classification. *Dis Esophagus* 2012; **25**: 235-241 [PMID: 21895852 DOI: 10.1111/j.1442-2050.2011.01241.x]

4 **Neurath MF**, Travis SP. Mucosal healing in inflammatory bowel diseases: a systematic review. *Gut* 2012; **61**: 1619-1635 [PMID: 22842618 DOI: 10.1136/gutjnl-2012-302830]

5 **Pineton de Chambrun G**, Peyrin-Biroulet L, Lémann M, Colombel JF. Clinical implications of mucosal healing for the management of IBD. *Nat Rev Gastroenterol Hepatol* 2010; **7**: 15-29 [PMID: 19949430 DOI: 10.1038/nrgastro.2009.203]

6 **Frøslie KF**, Jahnsen J, Moum BA, Vatn MH. Mucosal healing in inflammatory bowel disease: results from a Norwegian population-based cohort. *Gastroenterology* 2007; **133**: 412-422 [PMID: 17681162]

7 **Baert F**, Moortgat L, Van Assche G, Caenepeel P, Vergauwe P, De Vos M, Stokkers P, Hommes D, Rutgeerts P, Vermeire S, D'Haens G. Mucosal healing predicts sustained clinical remission in patients with early-stage Crohn's disease. *Gastroenterology* 2010; **138**: 463-468, quiz e10- e11 [PMID: 19818785 DOI: 10.1053/j.gastro.2009.09.056]

8 **Florén CH**, Benoni C, Willén R. Histologic and colonoscopic assessment of disease extension in ulcerative colitis. *Scand J Gastroenterol* 1987; **22**: 459-462 [PMID: 3602926]

9 **Kiesslich R**, Fritsch J, Holtmann M, Koehler HH, Stolte M, Kanzler S, Nafe B, Jung M, Galle PR, Neurath MF. Methylene blue-aided chromoendoscopy for the detection of intraepithelial neoplasia and colon cancer in ulcerative colitis. *Gastroenterology* 2003; **124**: 880-888 [PMID: 12671882 DOI: 10.1053/gast.2003.50146]

10 **Neumann H**, Vieth M, Atreya R, Neurath MF, Mudter J.

Prospective evaluation of the learning curve of confocal laser endomicroscopy in patients with IBD. *Histol Histopathol* 2011; **26**: 867-872 [PMID: 21630216]
11 **Watanabe O**, Ando T, Maeda O, Hasegawa M, Ishikawa D, Ishiguro K, Ohmiya N, Niwa Y, Goto H. Confocal endomicroscopy in patients with ulcerative colitis. *J Gastroenterol Hepatol* 2008; **23** Suppl 2: S286-S290 [PMID: 19120913 DOI: 10.1111/j.1440-1746.2008.05559.x]
12 **Li CQ**, Xie XJ, Yu T, Gu XM, Zuo XL, Zhou CJ, Huang WQ, Chen H, Li YQ. Classification of inflammation activity in ulcerative colitis by confocal laser endomicroscopy. *Am J Gastroenterol* 2010; **105**: 1391-1396 [PMID: 19935787]
13 **Neumann H**, Vieth M, Atreya R, Grauer M, Siebler J, Bernatik T, Neurath MF, Mudter J. Assessment of Crohn's disease activity by confocal laser endomicroscopy. *Inflamm Bowel Dis* 2012; **18**: 2261-2269 [PMID: 22344873 DOI: 10.1002/ibd.22907]
14 **Schmidt C**, Lautenschlaeger C, Collnot EM, Schumann M, Bojarski C, Schulzke JD, Lehr CM, Stallmach A. Nano- and microscaled particles for drug targeting to inflamed intestinal mucosa: a first in vivo study in human patients. *J Control Release* 2013; **165**: 139-145 [PMID: 23127508 DOI: 10.1016/j.jconrel.2012.10.019]
15 **Atreya R,** Neumann H, Neufert C, Waldner Mea, Neurath MF. In Vivo Molecular Imaging Using Fluorescent Anti-TNF Antibodies and Confocal LASER Endomicroscopy Predicts Response to Anti-TNF Therapy in Crohn's Disease. *Nat Med* 2013; In press
16 **Bessho R**, Kanai T, Hosoe N, Kobayashi T, Takayama T, Inoue N, Mukai M, Ogata H, Hibi T. Correlation between endocytoscopy and conventional histopathology in microstructural features of ulcerative colitis. *J Gastroenterol* 2011; **46**: 1197-1202 [PMID: 21805068 DOI: 10.1007/s00535-011-0439-1]
17 **Neumann H**, Vieth M, Neurath MF, Atreya R. Endocytoscopy allows accurate in vivo differentiation of mucosal inflammatory cells in IBD: a pilot study. *Inflamm Bowel Dis* 2013; **19**: 356-362 [PMID: 22644957 DOI: 10.1002/ibd.23025]
18 **Mescoli C**, Albertoni L, D'incá R, Rugge M. Dysplasia in inflammatory bowel diseases. *Dig Liver Dis* 2013; **45**: 186-194 [PMID: 22974564 DOI: 10.1016/j.dld.2012.07.013]
19 **Mattar MC**, Lough D, Pishvaian MJ, Charabaty A. Current management of inflammatory bowel disease and colorectal cancer. *Gastrointest Cancer Res* 2011; **4**: 53-61 [PMID: 21673876]
20 **Itzkowitz SH**, Harpaz N. Diagnosis and management of dysplasia in patients with inflammatory bowel diseases. *Gastroenterology* 2004; **126**: 1634-1648 [PMID: 15168373]
21 **Vagefi PA**, Longo WE. Colorectal cancer in patients with inflammatory bowel disease. *Clin Colorectal Cancer* 2005; **4**: 313-319 [PMID: 15663834]
22 **Müller AD**, Sonnenberg A. Prevention of colorectal cancer by flexible endoscopy and polypectomy. A case-control study of 32,702 veterans. *Ann Intern Med* 1995; **123**: 904-910 [PMID: 7486484]
23 **Collins PD**, Mpofu C, Watson AJ, Rhodes JM. Strategies for detecting colon cancer and/or dysplasia in patients with inflammatory bowel disease. *Cochrane Database Syst Rev* 2006; (2): CD000279 [PMID: 16625534 DOI: 10.1002/14651858.CD000279.pub3]
24 **van Rijn JC**, Reitsma JB, Stoker J, Bossuyt PM, van Deventer SJ, Dekker E. Polyp miss rate determined by tandem colonoscopy: a systematic review. *Am J Gastroenterol* 2006; **101**: 343-350 [PMID: 16454841 DOI: 10.1111/j.1572-0241.2006.00390.x]
25 **Rex DK**, Cutler CS, Lemmel GT, Rahmani EY, Clark DW, Helper DJ, Lehman GA, Mark DG. Colonoscopic miss rates of adenomas determined by back-to-back colonoscopies. *Gastroenterology* 1997; **112**: 24-28 [PMID: 8978338]
26 **Kaplan GG**, Heitman SJ, Hilsden RJ, Urbanski S, Myers RP, Lee SS, Burak KW, Swain M, Panaccione R. Population-based analysis of practices and costs of surveillance for colonic dysplasia in patients with primary sclerosing cholangitis and colitis. *Inflamm Bowel Dis* 2007; **13**: 1401-1407 [PMID: 17600816 DOI: 10.1002/ibd.20204]
27 **Hoffman A**, Goetz M, Vieth M, Galle PR, Neurath MF, Kiesslich R. Confocal laser endomicroscopy: technical status and current indications. *Endoscopy* 2006; **38**: 1275-1283 [PMID: 17163333 DOI: 10.1055/s-2006-944813]
28 **Goetz M**, Toermer T, Vieth M, Dunbar K, Hoffman A, Galle PR, Neurath MF, Delaney P, Kiesslich R. Simultaneous confocal laser endomicroscopy and chromoendoscopy with topical cresyl violet. *Gastrointest Endosc* 2009; **70**: 959-968 [PMID: 19595315 DOI: 10.1016/j.gie.2009.04.016]
29 **Kiesslich R**, Goetz M, Lammersdorf K, Schneider C, Burg J, Stolte M, Vieth M, Nafe B, Galle PR, Neurath MF. Chromoscopy-guided endomicroscopy increases the diagnostic yield of intraepithelial neoplasia in ulcerative colitis. *Gastroenterology* 2007; **132**: 874-882 [PMID: 17383417 DOI: 10.1053/j.gastro.2007.01.048]
30 **Hurlstone DP**, Thomson M, Brown S, Tiffin N, Cross SS, Hunter MD. Confocal endomicroscopy in ulcerative colitis: differentiating dysplasia-associated lesional mass and adenoma-like mass. *Clin Gastroenterol Hepatol* 2007; **5**: 1235-1241 [PMID: 17690019]
31 **van den Broek FJ**, van Es JA, van Eeden S, Stokkers PC, Ponsioen CY, Reitsma JB, Fockens P, Dekker E. Pilot study of probe-based confocal laser endomicroscopy during colonoscopic surveillance of patients with longstanding ulcerative colitis. *Endoscopy* 2011; **43**: 116-122 [PMID: 21165821 DOI: 10.1055/s-0030-1255954]
32 **Su P**, Liu Y, Lin S, Xiao K, Chen P, An S, He J, Bai Y. Efficacy of confocal laser endomicroscopy for discriminating colorectal neoplasms from non-neoplasms: a systematic review and meta-analysis. *Colorectal Dis* 2013; **15**: e1-e12 [PMID: 23006609 DOI: 10.1111/codi.12033]
33 **Jørgensen KK**, Grzyb K, Lundin KE, Clausen OP, Aamodt G, Schrumpf E, Vatn MH, Boberg KM. Inflammatory bowel disease in patients with primary sclerosing cholangitis: clinical characterization in liver transplanted and nontransplanted patients. *Inflamm Bowel Dis* 2012; **18**: 536-545 [PMID: 21456044 DOI: 10.1002/ibd.21699]
34 **Lindberg BU**, Broomé U, Persson B. Proximal colorectal dysplasia or cancer in ulcerative colitis. The impact of primary sclerosing cholangitis and sulfasalazine: results from a 20-year surveillance study. *Dis Colon Rectum* 2001; **44**: 77-85 [PMID: 11805567]
35 **Claessen MM**, Lutgens MW, van Buuren HR, Oldenburg B, Stokkers PC, van der Woude CJ, Hommes DW, de Jong DJ, Dijkstra G, van Bodegraven AA, Siersema PD, Vleggaar FP. More right-sided IBD-associated colorectal cancer in patients with primary sclerosing cholangitis. *Inflamm Bowel Dis* 2009; **15**: 1331-1336 [PMID: 19229982 DOI: 10.1002/ibd.20886]
36 **Dlugosz A,** Barakat AM, Ost A, Bergquist A. Probe-Based Confocal Laser Endomicroscopy in Colonoscopic Surveillance of Patients With PSC-IBD. *Gastroenterology* 2013; **144** (5(SUP1)): S87. Available from: URL: http://www.gastrojournal.org/supplements
37 **af Björkesten CG**, Nieminen U, Sipponen T, Turunen U, Arkkila P, Färkkilä M. Mucosal healing at 3 months predicts long-term endoscopic remission in anti-TNF-treated luminal Crohn's disease. *Scand J Gastroenterol* 2013; **48**: 543-551 [PMID: 23477356 DOI: 10.3109/00365521.2013.772230]
38 **Rutgeerts P**, Van Assche G, Sandborn WJ, Wolf DC, Geboes K, Colombel JF, Reinisch W, Kumar A, Lazar A, Camez A, Lomax KG, Pollack PF, D'Haens G. Adalimumab induces and maintains mucosal healing in patients with Crohn's disease: data from the EXTEND trial. *Gastroenterology* 2012; **142**: 1102-1111.e2 [PMID: 22326435 DOI: 10.1053/j.gastro.2012.01.035]
39 **Colombel JF**, Rutgeerts P, Reinisch W, Esser D, Wang Y, Lang Y, Marano CW, Strauss R, Oddens BJ, Feagan BG,

Hanauer SB, Lichtenstein GR, Present D, Sands BE, Sandborn WJ. Early mucosal healing with infliximab is associated with improved long-term clinical outcomes in ulcerative colitis. *Gastroenterology* 2011; **141**: 1194-1201 [PMID: 21723220]

40 **Kiesslich R**, Goetz M, Angus EM, Hu Q, Guan Y, Potten C, Allen T, Neurath MF, Shroyer NF, Montrose MH, Watson AJ. Identification of epithelial gaps in human small and large intestine by confocal endomicroscopy. *Gastroenterology* 2007; **133**: 1769-1778 [PMID: 18054549]

41 **Liu JJ**, Madsen KL, Boulanger P, Dieleman LA, Meddings J, Fedorak RN. Mind the gaps: confocal endomicroscopy showed increased density of small bowel epithelial gaps in inflammatory bowel disease. *J Clin Gastroenterol* 2011; **45**: 240-245 [PMID: 21030873 DOI: 10.1097/MCG.0b013e3181fbdb8a]

42 **Kiesslich R**, Duckworth CA, Moussata D, Gloeckner A, Lim LG, Goetz M, Pritchard DM, Galle PR, Neurath MF, Watson AJ. Local barrier dysfunction identified by confocal laser endomicroscopy predicts relapse in inflammatory bowel disease. *Gut* 2012; **61**: 1146-1153 [PMID: 22115910]

43 **Turcotte JF**, Wong K, Mah SJ, Dieleman LA, Kao D, Kroeker K, Claggett B, Saltzman JR, Wine E, Fedorak RN, Liu JJ. Increased epithelial gaps in the small intestine are predictive of hospitalization and surgery in patients with inflammatory bowel disease. *Clin Transl Gastroenterol* 2012; **3**: e19 [PMID: 23238291 DOI: 10.1038/ctg.2012.13]

44 **Liu JJ**, Rudzinski JK, Mah SJ, Thiesen AL, Bao H, Wine E, Ogg SC, Boulanger P, Fedorak RN, Madsen KL. Epithelial gaps in a rodent model of inflammatory bowel disease: a quantitative validation study. *Clin Transl Gastroenterol* 2011; **2**: e3 [PMID: 23237881 DOI: 10.1038/ctg.2011.2]

45 **Liu JJ**, Wong K, Thiesen AL, Mah SJ, Dieleman LA, Claggett B, Saltzman JR, Fedorak RN. Increased epithelial gaps in the small intestines of patients with inflammatory bowel disease: density matters. *Gastrointest Endosc* 2011; **73**: 1174-1180 [PMID: 21396639]

46 **Liu JJ**, Rudzinski JK, Mah SJ, Thiesen AL, Bao HY, Wine E, Ogg SC, Boulanger P, Fedorak RN, Madsen KL. Epithelial gaps in a rodent model of inflammatory bowel disease: a quantitative validation study. *Clin and Trans Gastro* 2011; **2**: e3 [PMID: 23237881]

47 **Montrose MH**. The future of GI and liver research: editorial perspectives: I. Visions of epithelial research. *Am J Physiol Gastrointest Liver Physiol* 2003; **284**: G547-G550 [PMID: 12631555 DOI: 10.1152/ajpgi.00547.2002]

48 **Clayburgh DR**, Shen L, Turner JR. A porous defense: the leaky epithelial barrier in intestinal disease. *Lab Invest* 2004; **84**: 282-291 [PMID: 14767487]

49 **Gill SR**, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM, Nelson KE. Metagenomic analysis of the human distal gut microbiome. *Science* 2006; **312**: 1355-1359 [PMID: 16741115]

50 **Neish AS**. Microbes in gastrointestinal health and disease. *Gastroenterology* 2009; **136**: 65-80 [PMID: 19026645]

51 **Watson AJ**, Duckworth CA, Guan Y, Montrose MH. Mechanisms of epithelial cell shedding in the Mammalian intestine and maintenance of barrier function. *Ann N Y Acad Sci* 2009; **1165**: 135-142 [PMID: 19538298]

52 **Ishiguro Y**. Mucosal proinflammatory cytokine production correlates with endoscopic activity of ulcerative colitis. *J Gastroenterol* 1999; **34**: 66-74 [PMID: 10204613]

53 **Monteleone G**, Trapasso F, Parrello T, Biancone L, Stella A, Iuliano R, Luzza F, Fusco A, Pallone F. Bioactive IL-18 expression is up-regulated in Crohn's disease. *J Immunol* 1999; **163**: 143-147 [PMID: 10384110]

54 **Moussata D**, Goetz M, Gloeckner A, Kerner M, Campbell B, Hoffman A, Biesterfeld S, Flourie B, Saurin JC, Galle PR, Neurath MF, Watson AJ, Kiesslich R. Confocal laser endomicroscopy is a new imaging modality for recognition of intramucosal bacteria in inflammatory bowel disease in vivo. *Gut* 2011; **60**: 26-33 [PMID: 20980342]

55 **Neumann H**, Günther C, Vieth M, Grauer M, Wittkopf N, Mudter J, Becker C, Schoerner C, Atreya R, Neurath MF. Confocal laser endomicroscopy for in vivo diagnosis of Clostridium difficile associated colitis - a pilot study. *PLoS One* 2013; **8**: e58753 [PMID: 23527018 DOI: 10.1371/journal.pone.0058753]

56 **May GR**, Sutherland LR, Meddings JB. Is small intestinal permeability really increased in relatives of patients with Crohn's disease? *Gastroenterology* 1993; **104**: 1627-1632 [PMID: 8500719]

57 **Meddings JB**. Review article: Intestinal permeability in Crohn's disease. *Aliment Pharmacol Ther* 1997; **11** Suppl 3: 47-53; discussion 53-6 [PMID: 9467978]

58 **Liu JJ**, Davis EM, Wine E, Lou Y, Rudzinski JK, Alipour M, Boulanger P, Thiesen AL, Sergi C, Fedorak RN, Muruve D, Madsen KL, Irvin RT. Epithelial cell extrusion leads to breaches in the intestinal epithelium. *Inflamm Bowel Dis* 2013; **19**: 912-921 [PMID: 23511029 DOI: 10.1097/MIB.0b013e3182807600]

P- Reviewers: Akyuz F, Afzal NA, Ciacci C, Ciccone MM
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.v19.i43.7552

World J Gastroenterol 2013 November 21; 19(43): 7552-7560
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Mucosal healing and deep remission: What does it mean?

Gerhard Rogler, Stephan Vavricka, Alain Schoepfer, Peter L Lakatos

Gerhard Rogler, Stephan Vavricka, Division of Gastroenterology and Hepatology, Department of Internal Medicine, University Hospital Zürich, 8091 Zürich, Switzerland
Gerhard Rogler, Stephan Vavricka, Zurich Center for Integrative Human Physiology, University of Zurich, 8006 Zürich, Switzerland
Stephan Vavricka, Gastroenterology, Triemli Spital, 8063 Zürich, Switzerland
Alain Schoepfer, Division of Gastroenterology and Hepatology, Centre Hospitalier Universitaire Vaudois et Université de Lausanne, 1011 Lausanne, Switzerland
Peter L Lakatos, Division of Gastroenterology and Hepatology, 1st Department of Medicine, Semmelweis University, H1083 Budapest, Hungary
Author contributions: Rogler G made the concept of the manuscript; Rogler G, Vavricka S, Schoepfer A and Lakatos PL wrote the manuscript together.
Supported by Grants from the Swiss National Science Foundation to Rogler G, Grant No. 310030-120312; to Schoepfer A, Grant No. 32003B_135665/1; to Vavricka S, Grant No. 320000-114009/3 and 32473B_135694/1; and to the Swiss IBD Cohort, Grant No. 33CS30_134274
Correspondence to: Gerhard Rogler, MD, PhD, Division of Gastroenterology and Hepatology, Department of Internal Medicine, University Hospital Zürich, Rämistrasse 100, 8091 Zürich, Switzerland. gerhard.rogler@usz.ch
Telephone: +41-44-2559519 Fax: +41-44-2559479
Received: July 15, 2013 Revised: September 20, 2013
Accepted: September 29, 2013
Published online: November 21, 2013

Abstract

The use of specific terms under different meanings and varying definitions has always been a source of confusion in science. When we point our efforts towards an evidence based medicine for inflammatory bowel diseases (IBD) the same is true: Terms such as "mucosal healing" or "deep remission" as endpoints in clinical trials or treatment goals in daily patient care may contribute to misconceptions if meanings change over time or definitions are altered. It appears to be useful to first have a look at the development of terms and their definitions, to assess their intrinsic and context-independent problems and then to analyze the different relevance in present-day clinical studies and trials. The purpose of such an attempt would be to gain clearer insights into the true impact of the clinical findings behind the terms. It may also lead to a better defined use of those terms for future studies. The terms "mucosal healing" and "deep remission" have been introduced in recent years as new therapeutic targets in the treatment of IBD patients. Several clinical trials, cohort studies or inception cohorts provided data that the long term disease course is better, when mucosal healing is achieved. However, it is still unclear whether continued or increased therapeutic measures will aid or improve mucosal healing for patients in clinical remission. Clinical trials are under way to answer this question. Attention should be paid to clearly address what levels of IBD activity are looked at. In the present review article authors aim to summarize the current evidence available on mucosal healing and deep remission and try to highlight their value and position in the everyday decision making for gastroenterologists.

Key words: Inflammatory bowel disease; Mucosal healing; Deep remission; Treatment targets; Clinical activity

Core tip: "Mucosal healing" and "deep remission" have been discussed heavily as "new" treatment goals in inflammatory bowel diseases patients in recent years. This was based on evidence that the long term disease behaviour appears to be better, when mucosal healing is achieved. Unfortunately, a definite proof that therapy escalation for patients in clinical remission not achieving mucosal healing will be beneficial is still lacking. Clinical trials are under way to answer this question. At the moment it appears to be helpful to summarize the current evidence available on mucosal healing and deep remission to support the everyday decision making for gastroenterologists.

Rogler G, Vavricka S, Schoepfer A, Lakatos PL. Mucosal healing and deep remission: What does it mean? *World J Gastroenterol*

2013; 19(43): 7552-7560 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7552.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7552

INTRODUCTION

Assessing the activity of inflammatory bowel disease (IBD) is important for our daily practice treating patients with these chronic inflammatory diseases. The assessment of disease activity will guide our therapeutic decision and our choice of medication. Furthermore it is most important for clinical investigations of new treatment options and new drugs. The reduction of disease activity remains the most important endpoint in clinical trials.

However, the discussion on which parameters are most useful for this purpose is still ongoing and unresolved.

Assessment of activity of IBD can be performed on different levels such as clinical activity, biochemical activity (*e.g.* by measuring CRP or fecal calprotectin), endoscopy, and histology. Clinical remission in a given IBD patient does not necessarily imply biochemical, endoscopic, or histologic remission. To evaluate biochemical, endoscopic, and histologic activity, an increasing degree of invasive measures (blood sample, endoscopy, biopsies) is required. Assessing activity in IBD has thereby analogies to the iceberg phenomenon where the clinical assessment on the surface may show clinical remission, but inflammatory activity may still be present on biochemical, endoscopic, and histologic level (Figure 1).

HISTOLOGICAL REMISSION AS INITIAL DEFINITION OF MUCOSAL HEALING

One of the first scientists and clinicians that used the term "healing" or "mucosal healing" within the field of IBD was Burton I. Korelitz, past chief of the Division of Gastroenterology at Lenox Hill Hospital in New York[1]. However, he used this term exclusively with respect to histological changes of the mucosa[1]. So when the term "mucosal healing" was introduced into IBD clinic it meant the absence of histological alterations of the mucosa. Korelitz was well aware that healing of IBD is not regarded to be possible as both Crohn's disease (CD) and ulcerative colitis (UC) are regarded to be chronic diseases without spontaneous healing[2]. There may be an absence of symptoms and flares over years but mucosal inflammation may re-occur after remission for years or even decades (Figure 1).

Histological healing is difficult to determine especially in Crohn's disease as the inflammation may be patchy and a biopsy could miss an inflammatory infiltrate only a few millimeters away[3]. Similarly, in UC the histological evaluation of a biopsy may be misleading[4]. Histological alterations may be absent from the rectum and sigmoid due to effective topical therapy despite the presence of inflammation further proximal in the colon that may not be obvious to the endoscopist[4,5]. Histological healing would mean that we have to be sure that there had been an inflammatory infiltrate at a specific localization that completely disappeared upon therapy (or spontaneously). As is obvious this is hard or even impossible to prove as this would require frequent endoscopies with many biopsy samples and a labeling of former biopsy locations. Due to the impracticability of this approach the overall acceptance of the concept of "histological healing" was very limited[5]. Of note, newer techniques such as endomicroscopy suffer from the same shortcomings.

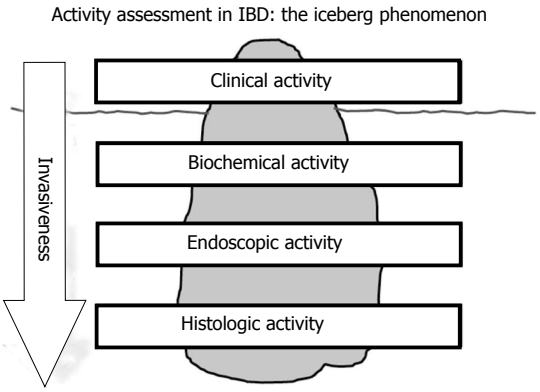


Figure 1 Activity assessment in inflammatory bowel disease: The iceberg phenomenon. IBD: inflammatory bowel disease.

ENDOSCOPIC REMISSION AS A NEW CONCEPT FOR MUCOSAL HEALING

In contrast to the initial concept of "mucosal healing" as a "disappearance of inflammatory infiltrate"[2] recent original manuscripts and reviews on the topic have used the term under different meanings. The "newer" meanings of "mucosal healing" have been summarized again by Korelitz in a critical review[2]. One of the "newer meanings" of mucosal healing would be the absence of inflammation ("healed mucosa") to the eye of the endoscopist, a definition that now has been applied in many clinical trials[6-16].

There is an obvious problem with this definition. One must assume the location of endoscopically normal mucosa has previously been inflamed[2]. Certainly this is easier to assess with endoscopy rather than histology as the area of evaluation is larger and small local differences and a patchy pattern would play a less important role. Nevertheless it requires that two endoscopical examinations are compared.

The definition also ignores that in endoscopically normal appearing mucosa there still may be histological inflammation. Another problem of this definition of course is that the inter-observer reproducibility of endoscopical IBD scores usually is very poor[17] and depends on the experience of the endoscopist[18] regardless of the technique used[19,20] (it may be discussed whether a kappa

between 0.7 and 0.8 is satisfying). Usually endoscopic findings are assessed on fixed point scales or described by dichotomous variables (present/absent)[18,21]. However, as outlined by de Lange and colleagues "endoscopic features of mucosal inflammation are continuous variables" for which dichotomous decisions are artificial and always require individual decisions[18]. The question arises how to interpret endoscopical findings indicating a clearly improved appearance of the mucosa in endoscopy with some or few remaining scattered erosions. A further important question arises with respect to endoscopical findings that cannot be interpreted as present inflammation but as residuals of former inflammation and a lack of complete normalization of the mucosa. Such findings would be pseudopolyps in an otherwise normal-appearing colon.

BIOCHEMICAL (FECAL MARKERS) REMISSION AS MUCOSAL HEALING

Fecal markers such as calprotectin or lactoferrin correlate very well with the degree and extent of infiltration of the mucosa by leukocytes. A good correlation between fecal calprotectin and the Crohn's Disease Endoscopic Index of Severity (CDEIS) was reported in several studies[22,23]. There is also a good correlation of fecal calprotectin with the Simple Endoscopic Score for Crohn's disease (SES-CD) which itself has a strong correlation with the CDEIS (correlation coefficient $r = 0.920$) and an excellent inter-observer reliability (κ coefficients 0.791-1.000)[24].

In ulcerative colitis calprotectin correlates well with disease activity as determined by histology and endoscopy[25,26].

It is a familiar experience to endoscopists that the mucosa may appear completely normal (healed) in patients that still have a markedly elevated fecal calprotectin. This would be an endoscopic remission but not biochemical remission, most likely reflecting a lack of histological remission with neutrophils still being present in the mucosal wall. It has been well established that calprotectin better correlates with histological findings (at least in UC) as compared to serum parameters or endoscopy[27-29].

MUCOSAL HEALING AND DEEP REMISSION: THE CONFUSED CLINICIAN

Surprisingly, some recent trials have reported a higher relative amount of patients with mucosal healing compared to the percentage of patients with clinical remission, especially in UC[30]. In those trials usually the endoscopist defined whether mucosal healing was present. How can this be explained? One reason could be that those patients had concomitant irritable bowel syndrome that was responsible for their complaints but no relevant remaining inflammation ("IBS superimposed on IBD"). The argument is straight forward and logical but it probably does not explain all cases. Firstly, little or no information is available on the histological remission in those patients. Histological remission - if evaluated by biopsies - again may be patchy and the evaluated biopsies may not be representative. Damage to deeper layers of the mucosa may have occurred that are not visible to the endoscopist's eye. Therefore is has to be challenged whether healed mucosa to the eye of the endoscopist is indeed the "most satisfying objective confirmation to support the clinical response" as outlined by Korelitz[2]. As he states the endoscopic healing "might be satisfactory for comparison in time for response to therapy in an individual case, but not for mucosal healing as an entity and certainly not to be used as an index of response to therapy in trials."[2]

To minimize the subjective component many clinical trials now apply the principle of a "central reader". Not only does this make trials more complicated, more expensive and more time consuming. It substitutes the problem of a bias introduced by many subjective evaluations of the mucosal response to a bias introduced by one subjective interpretation of findings. The intra-observer agreement for many endoscopic scores is not satisfactory. It may well be argued that the subjective criteria used by a central reader may not be accepted by others and that there could be a reduction of bias by a "multi-subjective" view (as we assume is the case for multicenter trials as compared to monocentric studies). Of note, in a recent randomized-controlled trial in patients with UC the conclusion was significantly changed after blinded central review of endoscopic images, suggesting that central reading of endoscopy may be necessary for regulatory purposes[31]. However, the question about the best method of objective endoscopic assessment is far from being answered.

Korelitz[2] suggested that histological healing should be the "minimal criterion for mucosal healing and preferably this information should be derived from multiple biopsy sites of previous inflammation". However, this would implicate that the evaluation of inflammation by a pathologist is objective. There have been studies on the inter-observer and intra-observer agreement of pathology findings[32]. Those results are not very encouraging. When a number of established criteria were used (excess of histiocytes in combination with a villous or irregular aspect of the mucosal surface and granulomas) experienced pathologists could correctly classify 70% of CD patients and 75% of UC patients[32]. Especially in mild disease, there is still dispute as to whether the presence of a "physiological (minor) inflammation" should be regarded as manifestation of IBD or not. Clinically unaffected siblings of IBD patients may show mild histological inflammation and increased cellular activation markers[33]. Cell counting will not solve the problem. The request for a "central pathology reader" also is not helpful as the same dilemma as for the central endoscopy reader will occur. Moreover, different pathologists have suggested different criteria to evaluate the presence or absence of "un-normal" inflammation (for an overview see[3,34-37]. There is no agreement on that. Geboes for ex-

ample suggested that the presence of neutrophils in the intestinal epithelium is an important discriminator for the presence or absence of inflammation. He therefore suggested that a combination of endoscopy and histology should be used to evaluate the presence of inflammation in IBD patients to finally judge whether mucosal healing has been achieved (see above).

MUCOSAL HEALING AND DEEP REMISSION: THE CONFUSED SCIENTIST

CD and UC are regarded to be chronic diseases that never disappear. The concept of a healing of a part of the body affected by such a disease subsequently is surprising for scientists working on the elucidation of the pathophysiology of IBD.

However, there is another aspect that is disturbing. There have been reports that even in macroscopically and microscopically normal appearing mucosa specific changes can be found that are characteristic for inflammation or at least changes that could be associated with the pathophysiology[38-45].

Changes of the microbiota in the lumen of the gut have been described in IBD patients despite the absence of detectable inflammation[46-51]. Could a "complete deep remission" be possible without normalization of the intestinal microbiome? The mucus layer of the mucosa may be changed also in normal appearing mucosa in endoscopy[52-56]. The normal fixation procedure of biopsies and the subsequent H&E staining does not allow evaluation of the mucus layer as it is destroyed during this procedure. A reduced thickness of the mucus layer in UC in remission has been described[54,56,57] as well as a reduced secretion of mucin[52,53,58-60] or defensins[61-64]. The question arises whether the mucosa can be termed as "normal" or "healed" if those changes are still present.

Epithelial cells may have an impaired barrier function despite a lack of inflammatory signs. Cytokine expression and cytokine secretion by immune cells may still be significantly increased despite a normal appearing histology. A normalization of those changes has been termed biochemical healing[65-68]. There are no data available with respect to the predictive value of "biochemical healing" and whether this would correlate to a more favorable disease outcome.

The confused scientist, however, is able to imagine a further level of "healing". In macroscopically normal appearing mucosa with microscopically normal appearing cells that display normal cytokine expression and secretion levels, epigenetic changes may still be present that may trigger pathological responses upon minor stimuli[69-76]. Can a persistence of epigenetic changes in otherwise normal mucosa be termed "mucosal healing"? Or do we have to achieve "epigenetic healing" to finally achieve the best outcome possible for our patients? These questions will have to be answered in the future. Currently we are just at the start of investigations into these aspects with the first interesting pieces of the puzzle being put together.

MUCOSAL HEALING AND DEEP REMISSION: THE CONFUSED "TRIALIST"

As mentioned above the terms "mucosal healing" and "deep remission" have been used in a number of trials with quite different meanings and definitions. The key confounder is the lack of unequivocal definition(s). Therefore, results and data from those trials with respect to mucosal healing cannot easily be compared. Nevertheless, this is done frequently. In most cases endoscopical investigation is used for the evaluation of "mucosal healing". One crucial point is whether "mucosal healing" was defined simply as the absence of ulcers when ulcers had been seen previously or whether the absence of ulcerations and ulcers was investigated exactly at a place where those alterations had been found before.

The above is reflected in the way different trials have been reported. In the ACCENT 1 endoscopic substudy the CDEIS was used for scoring and the complete absence of mucosal ulcerations that were observed at baseline was evaluated[77]. In the SONIC study in contrast no clearly defined score was used. Mucosal healing was defined as "complete absence of mucosal ulceration in the colon and terminal ileum"[78]. In the "Top-down versus step up" study by Gert D'Haens and coworkers SES-CD was used for the evaluation of mucosal healing which was a secondary endpoint[79,80]. Mucosal healing was defined as "absence of ulcers". In the MUSIC trials again the CDEIS was applied. The definition of mucosal healing was "absence of ulcers and endoscopic remission defined as CDEIS < 6". In the EXTEND study applying again SES-CD mucosal healing was seen as "absence of mucosal ulceration"[81]. As is obvious from those definitions, the question arises whether a few remaining aphthous lesions in a patient with severe and deep ulcers at the beginning of therapy also may be termed mucosal healing.

For UC the IOIBD attempted a consensus for mucosal healing in 2007: "absence of friability, blood, erosions and ulcers in all visualized segments of the gut mucosa". According to the IOIBD experts the presence of an abnormal vascular pattern is still compatible with mucosal healing or "normal mucosa". However, also in UC the definitions applied varied widely: In the ACT1 study mucosal healing was a secondary endpoint[82,83]. The Mayo endoscopic subscore was used and mucosal healing was defined as "absolute subscore for endoscopy of 0 or 1"[82,83]. The same definition was used for ULTRA 2[84].

In studies on the outcome of therapy with 5-aminosalicylic acid the definition of mucosal healing largely defined the number of patients achieving this endpoint (Table 1). As an example, Vecchi *et al*[85] compared mesalazine 4 g orally *vs* 2 + 2 g orally and enema in 2001 in patients with a clinical activity index (CAI) of 4-12 and used an endoscopic Rachmilewitz index < 4 as definition of mucosal healing leading to 58% *vs* 71% of patients

Table 1 Association between the definitions of remission and mucosal healing and actual healing rates in patients with ulcerative colitis treated with mesalazine

Author	Design	Study	Timing of endoscopy	Endoscopic index	Def. of MH	No of pat. Achieving MH
Vecchi (2001)	Mc, RCT	Mesalazine 4 g orally *vs* 2 + 2 g orally and enema	6 wk	Rachmilewitz	Rachmilewitz < 4	58% *vs* 71%
Malchow (2002)	Mc, db, RCT	Mesalazine 4 g enema *vs* 1 g foam	4 wk	Rachmilewitz	Rachmilewitz < 2	38% *vs* 37%
Mansfield (2002)	Mc, db, RCT	Balsalazide 6.75 g *vs* sulfasal. 3g	8 wk	4 point scale	Score of 0 = normal mucosa	27% *vs* 25%
Hanauer (2007) Ascend	Mc, db, RCT	Asacol 4.8 g *vs* 2.4 g	6 wk	Descriptive, no score	Normal endoscopic finding	25% *vs* 20%
Kamm (2007) MMX	Mc, db, RCT	MMX mes. 4.8 g *vs* 2.4 g *vs* placebo	8 wk	Mod. Sutherland index	Mod Sutherland index < 1	77% *vs* 69% *vs* 46%
Kruis (2009)	Mc, db, RCT	Mesalazine 3 g *vs* 1g x 3	8 wk	Rachmilewitz	Rachmilewitz < 4	71% *vs* 70%

Mc: Multicenter; db: Double-blind; RCT: Randomized controlled trial; MH: Mesalazine.

Table 2 One of the problems in endoscopic ulcerative colitis scores is the application of varying criteria

	Truelove	Baron	Powell-T (St Mark's)	Levine	Rach-milewitz	Modified Baron	Mayo	Sutherland
Erythema						+	+	
Edema				+				
Granularity				+	+	+		
Vascular pattern		+		+	+	+	+	
Friability	+	+	+	+	+	+	+	+
Erosions				+	+		+	
Ulceration				+	+	+	+	
Exudate					+			+
Remission	0	0-1			0-2	0-1	0-1	0

achieving this endpoint[85]. In 2002 Malchow compared Mesalazine 4g enema *vs* 1g foam preparation in patients with a CAI > 4 for 4 wk and applied an endoscopic Rachmilewitz index < 2 as definition of mucosal healing leading to rates of 38% *vs* 37%[86]. As one would expect, the different definitions used cause huge variation in defined endoscopic mucosal healing rates in patients with UC, which makes the comparison of efficacy of different drugs or formulations extremely difficult.

One of the problems in endoscopic UC scores is the application of varying criteria (see Table 2). The reasons for such different definitions and endpoints may only be speculated. Unfortunately we lack an unequivocal definition; all of the scoring systems published so far have certain limitations, which have led to the introduction of several additional scoring systems. From a patient's and physician's perspective, however, the use of one single scoring system would be most desirable to enable valid comparisons among study outcomes.

WHAT IS THE ADDITIVE VALUE OF DEEP REMISSION AS COMPARED TO MUCOSAL HEALING?

"Deep remission" is another term that has been discussed as a treatment target in recent years. The definition, however, is unfortunately not clearer than the one of mucosal healing. In the EXTEND study "deep remission" was defined as clinical remission (CDAI < 150) and complete mucosal healing as defined according to CDEIS[13]. It is worthwhile to look a bit closer at this definition. If a patient with CD achieves mucosal healing but still has increased CDAI (no clinical remission) this may be due to superimposed IBS symptoms or the fact that without the presence of inflammation there is some bowel damage such as a fibrotic stricture or an internal fistula which might contribute to increased bowel frequency. Subsequently the lack of clinical remission is important for the patient and his/her clinical management (*e.g.* surgery of the stricture) but not for the medical (anti-inflammatory) management of the disease. Thus, the term "deep remission" in the definition outlined above is not useful and does not provide more information than mucosal healing. In fact - it contributes to confusion of scientists, clinicians and "trialists".

HOW CAN WE IMPROVE?

There should be standards on the definition of mucosal healing for clinical studies. It needs to be discussed - and finally decided - whether endoscopic mucosal healing, histologic mucosal healing or a combination of both can be standardized. Once agreement on definitions has been achieved, a given patient could be assessed by a -hopefully- simple binary coded tool that is oriented according to the TNM classification of oncology. A proposal for such a tool is illustrated in Table 3. The number "1"

Table 3 Proposal of the CBEHI classification to assess Crohn's disease activity

Activity level	Definition	Code
Clinical activity	Remission: CDAI < 150	C0
	Active: CDAI ⩾ 150	C1
Biochemical activity	CRP normal	B0 (CRP)
	Elevated CRP	B1 (CRP)
	Calprotectin < 200 μg/g	B0 (Calpro)
	Calprotectin ⩾ 200 μg/g	B1 (Calpro)
Endoscopic activity	Remission: SES-CD < 4	E0
	Active: SES-CD ⩾ 4	E1
Histologic activity	Inactive	H0
	Active	H1
Imaging	Inactive: no fistulas, no stenoses	I0
	Active: presence of either fistula and/or stenosis	I1

Example: A Crohn's disease (CD) patient with C0B0 (CRP) E1H1I0 would have clinical and biochemical remission, but endoscopic and histologic activity.

stands for "active", "0" for "remission" and "x" for "not assessed". Of note CD activity assessment would require, in contrast to UC, not only measuring clinical activity, biochemical, endoscopic and histologic activity, but also imaging modalities (presence of fistulas, strictures). This simple approach has the potential to reduce the amount of potentially confusing new definitions to describe different combinations of activities in IBD.

Other definitions of mucosal healing (such as "biological mucosal healing", "epigenetic mucosal healing", "mucus layer healing" or "microbiota mucosal healing") require further studies and prospective trials. At this point they are purely investigational and should not be used in clinical trials.

What would happen if such an agreement cannot be achieved? Then it would not make sense to discuss mucosal healing as a treatment target for IBD any further as this would be a treatment target that lacks a definition and subsequently is blurry, vague and indistinct.

ACKNOWLEDGMENTS

Gerhard Rogler has received in the last 2 years consultant fees from Abbott Switzerland and Abbott International, Tillotts International, FALK Germany, Essex/MSD Switzerland, Novartis, Roche, and Vifor Switzerland; GR has received speaker's honoraria from Abbott, FALK, MSD, Phadia, Tillotts, UCB, and Vifor; GR has received educational grants and research grants from Abbott, Ardeypharm, Essex/MSD, FALK, Flamentera, Novartis, Tillotts, UCB and Zeller.

Stephan Vavricka has received in the last 2 years consultant fees from Abbvie, MSD, and UCB, and Tillotts. SV has received speaker's honoraria from Abbott, MSD, Tillotts, UCB, and Vifor; SV has received educational grants and research grants from Abbot/Abbvie, Essex/MSD and UCB.

Alain Schoepfer has received in the last 2 years consultant fees from Abbvie, MSD, UCB, and Tillotts. AS has received speaker's honoraria from Abbvie; AS has received educational grants and research grants from Essex/MSD, UCB, and Tillotts.

Peter L Lakatos has received in the last 2 years consultant and lecture fees from Abbott/Abbvie, MSD-Hungary, and Ferring

REFERENCES

1 **Korelitz BI**, Sommers SC. Response to drug therapy in Crohn's disease: evaluation by rectal biopsy and mucosal cell counts. *J Clin Gastroenterol* 1984; **6**: 123-127 [PMID: 6143776]
2 **Korelitz BI**. Mucosal healing as an index of colitis activity: back to histological healing for future indices. *Inflamm Bowel Dis* 2010; **16**: 1628-1630 [PMID: 20803700 DOI: 10.1002/ibd.21268]
3 **Geboes K**. Is histology useful for the assessment of the efficacy of immunosuppressive agents in IBD and if so, how should it be applied ? *Acta Gastroenterol Belg* 2004; **67**: 285-289 [PMID: 15587337]
4 **Levine TS**, Tzardi M, Mitchell S, Sowter C, Price AB. Diagnostic difficulty arising from rectal recovery in ulcerative colitis. *J Clin Pathol* 1996; **49**: 319-323 [PMID: 8655709]
5 **Kane S**, Lu F, Kornbluth A, Awais D, Higgins PD. Controversies in mucosal healing in ulcerative colitis. *Inflamm Bowel Dis* 2009; **15**: 796-800 [PMID: 19213060 DOI: 10.1002/ibd.20875]
6 **Baert F**, Moortgat L, Van Assche G, Caenepeel P, Vergauwe P, De Vos M, Stokkers P, Hommes D, Rutgeerts P, Vermeire S, D'Haens G. Mucosal healing predicts sustained clinical remission in patients with early-stage Crohn's disease. *Gastroenterology* 2010; **138**: 463-468; quiz 463-468; [PMID: 19818785]
7 **Barreiro-de Acosta M**, Lorenzo A, Mera J, Dominguez-Muñoz JE. Mucosal healing and steroid-sparing associated with infliximab for steroid-dependent ulcerative colitis. *J Crohns Colitis* 2009; **3**: 271-276 [PMID: 21172286]
8 **Colombel JF**, Rutgeerts P, Reinisch W, Esser D, Wang Y, Lang Y, Marano CW, Strauss R, Oddens BJ, Feagan BG, Hanauer SB, Lichtenstein GR, Present D, Sands BE, Sandborn WJ. Early mucosal healing with infliximab is associated with improved long-term clinical outcomes in ulcerative colitis. *Gastroenterology* 2011; **141**: 1194-1201 [PMID: 21723220]
9 **Laharie D**, Reffet A, Belleannée G, Chabrun E, Subtil C, Razaire S, Capdepont M, de Lédinghen V. Mucosal healing with methotrexate in Crohn's disease: a prospective comparative study with azathioprine and infliximab. *Aliment Pharmacol Ther* 2011; **33**: 714-721 [PMID: 21235604 DOI: 10.1111/j.1365-2036.2010.04569.x]
10 **Levy LC**, Siegel CA. Endoscopic mucosal healing in ulcerative colitis. *Aliment Pharmacol Ther* 2011; **33**: 1254-1255; author reply 1254-1255 [PMID: 21535054 DOI: 10.1111/j.1365-2036.2011.04615.x]
11 **López-Palacios N**, Mendoza JL, Taxonera C, Lana R, López-

Jamar JM, Díaz-Rubio M. Mucosal healing for predicting clinical outcome in patients with ulcerative colitis using thiopurines in monotherapy. *Eur J Intern Med* 2011; **22**: 621-625 [PMID: 22075292]
12 **Mantzaris GJ**, Christidou A, Sfakianakis M, Roussos A, Koilakou S, Petraki K, Polyzou P. Azathioprine is superior to budesonide in achieving and maintaining mucosal healing and histologic remission in steroid-dependent Crohn's disease. *Inflamm Bowel Dis* 2009; **15**: 375-382 [PMID: 19009634 DOI: 10.1002/ibd.20777]
13 **Rutgeerts P**, Van Assche G, Sandborn WJ, Wolf DC, Geboes K, Colombel JF, Reinisch W, Kumar A, Lazar A, Camez A, Lomax KG, Pollack PF, D'Haens G. Adalimumab induces and maintains mucosal healing in patients with Crohn's disease: data from the EXTEND trial. *Gastroenterology* 2012; **142**: 1102-1111.e2 [PMID: 22326435]
14 **Lee KM**, Jeen YT, Cho JY, Lee CK, Koo JS, Park DI, Lim JP, Park SJ, Kim YS, Kim TO, Lee SH, Jang BI, Kim JW, Park YS, Kim ES, Choi CH, Kim HJ; IBD study Group of Korean Association for the Study of Intestinal Diseases. Efficacy, Safety, and Predictors of Response to Infliximab Therapy for Ulcerative Colitis: a Korean Multicenter Retrospective Study. *J Gastroenterol Hepatol* 2013 [PMID: 23829336 DOI: 10.1111/jgh.12324]
15 **Tursi A**, Elisei W, Giorgetti GM, Penna A, Picchio M, Brandimarte G. Factors Influencing Mucosal Healing in Crohn's Disease during Infliximab Treatment. *Hepatogastroenterology* 2013; **60**: 1041-1046 [PMID: 23803367 DOI: 10.5754/hge11514]
16 **Yokoyama K**, Kobayashi K, Mukae M, Sada M, Koizumi W. Clinical Study of the Relation between Mucosal Healing and Long-Term Outcomes in Ulcerative Colitis. *Gastroenterol Res Pract* 2013; **2013**: 192794 [PMID: 23762033 DOI: 10.1155/2013/192794]
17 **Orlandi F**, Brunelli E, Feliciangeli G, Svegliati-Baroni G, Di Sario A, Benedetti A, Guidarelli C, Macarri G. Observer agreement in endoscopic assessment of ulcerative colitis. *Ital J Gastroenterol Hepatol* 1998; **30**: 539-541 [PMID: 9836114]
18 **de Lange T**, Larsen S, Aabakken L. Inter-observer agreement in the assessment of endoscopic findings in ulcerative colitis. *BMC Gastroenterol* 2004; **4**: 9 [PMID: 15149550 DOI: 10.1186/1471-230X-4-9]
19 **Chen HB**, Huang Y, Chen SY, Deng DY, Gao LH, Xie JT, Yang LN, Huang C, He S, Li XL. A comparative study of two kinds of small bowel cleaning score system for capsule endoscopy. *Acta Gastroenterol Belg* 2012; **75**: 342-348 [PMID: 23082706]
20 **Bessho R**, Kanai T, Hosoe N, Kobayashi T, Takayama T, Inoue N, Mukai M, Ogata H, Hibi T. Correlation between endocytoscopy and conventional histopathology in microstructural features of ulcerative colitis. *J Gastroenterol* 2011; **46**: 1197-1202 [PMID: 21805068 DOI: 10.1007/s00535-011-0439-1]
21 **Travis SP**, Schnell D, Krzeski P, Abreu MT, Altman DG, Colombel JF, Feagan BG, Hanauer SB, Lémann M, Lichtenstein GR, Marteau PR, Reinisch W, Sands BE, Yacyshyn BR, Bernhardt CA, Mary JY, Sandborn WJ. Developing an instrument to assess the endoscopic severity of ulcerative colitis: the Ulcerative Colitis Endoscopic Index of Severity (UCEIS). *Gut* 2012; **61**: 535-542 [PMID: 21997563 DOI: 10.1136/gutjnl-2011-300486]
22 **Sipponen T**, Kärkkäinen P, Savilahti E, Kolho KL, Nuutinen H, Turunen U, Färkkilä M. Correlation of faecal calprotectin and lactoferrin with an endoscopic score for Crohn's disease and histological findings. *Aliment Pharmacol Ther* 2008; **28**: 1221-1229 [PMID: 18752630]
23 **Sipponen T**, Savilahti E, Kolho KL, Nuutinen H, Turunen U, Färkkilä M. Crohn's disease activity assessed by fecal calprotectin and lactoferrin: correlation with Crohn's disease activity index and endoscopic findings. *Inflamm Bowel Dis* 2008; **14**: 40-46 [PMID: 18022866 DOI: 10.1002/ibd.20312]
24 **Schoepfer AM**, Beglinger C, Straumann A, Trummler M, Vavricka SR, Bruegger LE, Seibold F. Fecal calprotectin correlates more closely with the Simple Endoscopic Score for Crohn's disease (SES-CD) than CRP, blood leukocytes, and the CDAI. *Am J Gastroenterol* 2010; **105**: 162-169 [PMID: 19755969]
25 **Røseth AG**, Aadland E, Grzyb K. Normalization of faecal calprotectin: a predictor of mucosal healing in patients with inflammatory bowel disease. *Scand J Gastroenterol* 2004; **39**: 1017-1020 [PMID: 15513345]
26 **Røseth AG**, Aadland E, Jahnsen J, Raknerud N. Assessment of disease activity in ulcerative colitis by faecal calprotectin, a novel granulocyte marker protein. *Digestion* 1997; **58**: 176-180 [PMID: 9144308]
27 **Vieira A**, Fang CB, Rolim EG, Klug WA, Steinwurz F, Rossini LG, Candelária PA. Inflammatory bowel disease activity assessed by fecal calprotectin and lactoferrin: correlation with laboratory parameters, clinical, endoscopic and histological indexes. *BMC Res Notes* 2009; **2**: 221 [PMID: 19874614 DOI: 10.1186/1756-0500-2-221]
28 **Licata A**, Randazzo C, Cappello M, Calvaruso V, Butera G, Florena AM, Peralta S, Cammà C, Craxì A. Fecal calprotectin in clinical practice: a noninvasive screening tool for patients with chronic diarrhea. *J Clin Gastroenterol* 2012; **46**: 504-508 [PMID: 22565607 DOI: 10.1097/MCG.0b013e318248f289]
29 **Kok L**, Elias SG, Witteman BJ, Goedhard JG, Muris JW, Moons KG, de Wit NJ. Diagnostic accuracy of point-of-care fecal calprotectin and immunochemical occult blood tests for diagnosis of organic bowel disease in primary care: the Cost-Effectiveness of a Decision Rule for Abdominal Complaints in Primary Care (CEDAR) study. *Clin Chem* 2012; **58**: 989-998 [PMID: 22407858 DOI: 10.1373/clinchem.2011.177980]
30 **Sandborn WJ**, van Assche G, Reinisch W, Colombel JF, D'Haens G, Wolf DC, Kron M, Tighe MB, Lazar A, Thakkar RB. Adalimumab induces and maintains clinical remission in patients with moderate-to-severe ulcerative colitis. *Gastroenterology* 2012; **142**: 257-265.e1-3 [PMID: 22062358 DOI: 10.1053/j.gastro.2011.10.032]
31 **Feagan BG**, Sandborn WJ, D'Haens G, Pola S, McDonald JW, Rutgeerts P, Munkholm P, Mittmann U, King D, Wong CJ, Zou G, Donner A, Shackelton LM, Gilgen D, Nelson S, Vandervoort MK, Fahmy M, Loftus EV, Panaccione R, Travis SP, Van Assche GA, Vermeire S, Levesque BG. The role of centralized reading of endoscopy in a randomized controlled trial of mesalamine for ulcerative colitis. *Gastroenterology* 2013; **145**: 149-157.e2 [PMID: 23528626 DOI: 10.1053/j.gastro.2013.03.025]
32 **Seldenrijk CA**, Morson BC, Meuwissen SG, Schipper NW, Lindeman J, Meijer CJ. Histopathological evaluation of colonic mucosal biopsy specimens in chronic inflammatory bowel disease: diagnostic implications. *Gut* 1991; **32**: 1514-1520 [PMID: 1773958]
33 **Zhulina Y**, Hahn-Strömberg V, Shamikh A, Peterson CG, Gustavsson A, Nyhlin N, Wickbom A, Bohr J, Bodin L, Tysk C, Carlson M, Halfvarson J. Subclinical inflammation with increased neutrophil activity in healthy twin siblings reflect environmental influence in the pathogenesis of inflammatory bowel disease. *Inflamm Bowel Dis* 2013; **19**: 1725-1731 [PMID: 23669399 DOI: 10.1097/MIB.0b013e318281f2d3]
34 **Geboes K**, Dalle I. Influence of treatment on morphological features of mucosal inflammation. *Gut* 2002; **50** Suppl 3: III37-III42 [PMID: 11953331]
35 **Bressenot A**, Geboes K, Vignaud JM, Guéant JL, Peyrin-Biroulet L. Microscopic features for initial diagnosis and disease activity evaluation in inflammatory bowel disease. *Inflamm Bowel Dis* 2013; **19**: 1745-1752 [PMID: 23782793 DOI: 10.1097/MIB.0b013e318281f2e8]
36 **Geboes K**, Colombel JF, Greenstein A, Jewell DP, Sandborn WJ, Vatn MH, Warren B, Riddell RH. Indeterminate colitis: a review of the concept--what's in a name? *Inflamm Bowel Dis* 2008; **14**: 850-857 [PMID: 18213696 DOI: 10.1002/ibd.20361]

37 **Geboes K**, Villanacci V. Terminology for the diagnosis of colitis. *J Clin Pathol* 2005; **58**: 1133-1134 [PMID: 16254099]
38 **Zamora SA**, Hilsden RJ, Meddings JB, Butzner JD, Scott RB, Sutherland LR. Intestinal permeability before and after ibuprofen in families of children with Crohn's disease. *Can J Gastroenterol* 1999; **13**: 31-36 [PMID: 10099814]
39 **Fries W**, Renda MC, Lo Presti MA, Raso A, Orlando A, Oliva L, Giofré MR, Maggio A, Mattaliano A, Macaluso A, Cottone M. Intestinal permeability and genetic determinants in patients, first-degree relatives, and controls in a high-incidence area of Crohn's disease in Southern Italy. *Am J Gastroenterol* 2005; **100**: 2730-2736 [PMID: 16393227]
40 **Secondulfo M**, de Magistris L, Fiandra R, Caserta L, Belletta M, Tartaglione MT, Riegler G, Biagi F, Corazza GR, Carratù R. Intestinal permeability in Crohn's disease patients and their first degree relatives. *Dig Liver Dis* 2001; **33**: 680-685 [PMID: 11785714]
41 **Hollander D**. Intestinal permeability in patients with Crohn's disease and their relatives. *Dig Liver Dis* 2001; **33**: 649-651 [PMID: 11785707]
42 **Peeters M**, Geypens B, Claus D, Nevens H, Ghoos Y, Verbeke G, Baert F, Vermeire S, Vlietinck R, Rutgeerts P. Clustering of increased small intestinal permeability in families with Crohn's disease. *Gastroenterology* 1997; **113**: 802-807 [PMID: 9287971]
43 **Howden CW**, Gillanders I, Morris AJ, Duncan A, Danesh B, Russell RI. Intestinal permeability in patients with Crohn's disease and their first-degree relatives. *Am J Gastroenterol* 1994; **89**: 1175-1176 [PMID: 8053430]
44 **Munkholm P**, Langholz E, Hollander D, Thornberg K, Orholm M, Katz KD, Binder V. Intestinal permeability in patients with Crohn's disease and ulcerative colitis and their first degree relatives. *Gut* 1994; **35**: 68-72 [PMID: 8307453]
45 **Hollander D**. Permeability in Crohn's disease: altered barrier functions in healthy relatives? *Gastroenterology* 1993; **104**: 1848-1851 [PMID: 8500744]
46 **Midtvedt T**, Zabarovsky E, Norin E, Bark J, Gizatullin R, Kashuba V, Ljungqvist O, Zabarovska V, Möllby R, Ernberg I. Increase of faecal tryptic activity relates to changes in the intestinal microbiome: analysis of Crohn's disease with a multidisciplinary platform. *PLoS One* 2013; **8**: e66074 [PMID: 23840402 DOI: 10.1371/journal.pone.0066074]
47 **Pérez-Brocal V**, García-López R, Vázquez-Castellanos JF, Nos P, Beltrán B, Latorre A, Moya A. Study of the viral and microbial communities associated with Crohn's disease: a metagenomic approach. *Clin Transl Gastroenterol* 2013; **4**: e36 [PMID: 23760301 DOI: 10.1038/ctg.2013.9]
48 **Papa E**, Docktor M, Smillie C, Weber S, Preheim SP, Gevers D, Giannoukos G, Ciulla D, Tabbaa D, Ingram J, Schauer DB, Ward DV, Korzenik JR, Xavier RJ, Bousvaros A, Alm EJ. Non-invasive mapping of the gastrointestinal microbiota identifies children with inflammatory bowel disease. *PLoS One* 2012; **7**: e39242 [PMID: 22768065 DOI: 10.1371/journal.pone.0039242]
49 **Varela E**, Manichanh C, Gallart M, Torrejón A, Borruel N, Casellas F, Guarner F, Antolin M. Colonisation by Faecalibacterium prausnitzii and maintenance of clinical remission in patients with ulcerative colitis. *Aliment Pharmacol Ther* 2013; **38**: 151-161 [PMID: 23725320 DOI: 10.1111/apt.12365]
50 **Vigsnaes LK**, van den Abbeele P, Sulek K, Frandsen HL, Steenholdt C, Brynskov J, Vermeiren J, van de Wiele T, Licht TR. Microbiotas from UC patients display altered metabolism and reduced ability of LAB to colonize mucus. *Sci Rep* 2013; **3**: 1110 [PMID: 23346367 DOI: 10.1038/srep01110]
51 **Nemoto H**, Kataoka K, Ishikawa H, Ikata K, Arimochi H, Iwasaki T, Ohnishi Y, Kuwahara T, Yasutomo K. Reduced diversity and imbalance of fecal microbiota in patients with ulcerative colitis. *Dig Dis Sci* 2012; **57**: 2955-2964 [PMID: 22623042 DOI: 10.1007/s10620-012-2236-y]
52 **Johansson ME**, Sjövall H, Hansson GC. The gastrointestinal mucus system in health and disease. *Nat Rev Gastroenterol Hepatol* 2013; **10**: 352-361 [PMID: 23478383 DOI: 10.1038/nrgastro.2013.35]
53 **Johansson ME**, Gustafsson JK, Holmén-Larsson J, Jabbar KS, Xia L, Xu H, Ghishan FK, Carvalho FA, Gewirtz AT, Sjövall H, Hansson GC. Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis. *Gut* 2013 [PMID: 23426893]
54 **Fyderek K**, Strus M, Kowalska-Duplaga K, Gosiewski T, Wedrychowicz A, Jedynak-Wasowicz U, Sładek M, Pieczarkowski S, Adamski P, Kochan P, Heczko PB. Mucosal bacterial microflora and mucus layer thickness in adolescents with inflammatory bowel disease. *World J Gastroenterol* 2009; **15**: 5287-5294 [PMID: 19908336]
55 **Braun A**, Treede I, Gotthardt D, Tietje A, Zahn A, Ruhwald R, Schoenfeld U, Welsch T, Kienle P, Erben G, Lehmann WD, Fuellekrug J, Stremmel W, Ehehalt R. Alterations of phospholipid concentration and species composition of the intestinal mucus barrier in ulcerative colitis: a clue to pathogenesis. *Inflamm Bowel Dis* 2009; **15**: 1705-1720 [PMID: 19504612 DOI: 10.1002/ibd.20993]
56 **Swidsinski A**, Loening-Baucke V, Theissig F, Engelhardt H, Bengmark S, Koch S, Lochs H, Dörffel Y. Comparative study of the intestinal mucus barrier in normal and inflamed colon. *Gut* 2007; **56**: 343-350 [PMID: 16908512]
57 **Pullan RD**, Thomas GA, Rhodes M, Newcombe RG, Williams GT, Allen A, Rhodes J. Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis. *Gut* 1994; **35**: 353-359 [PMID: 8150346]
58 **Habib NA**, Dawson PM, Krausz T, Blount MA, Kersten D, Wood CB. A study of histochemical changes in mucus from patients with ulcerative colitis, Crohn's disease, and diverticular disease of the colon. *Dis Colon Rectum* 1986; **29**: 15-17 [PMID: 3940799]
59 **Dorofeyev AE**, Vasilenko IV, Rassokhina OA, Kondratiuk RB. Mucosal barrier in ulcerative colitis and Crohn's disease. *Gastroenterol Res Pract* 2013; **2013**: 431231 [PMID: 23737764 DOI: 10.1155/2013/431231]
60 **Sperber K**, Shim J, Mehra M, Lin A, George I, Ogata S, Mayer L, Itzkowitz S. Mucin secretion in inflammatory bowel disease: comparison of a macrophage-derived mucin secretagogue (MMS-68) to conventional secretagogues. *Inflamm Bowel Dis* 1998; **4**: 12-17 [PMID: 9552223]
61 **Wehkamp J**, Stange EF, Fellermann K. Defensin-immunology in inflammatory bowel disease. *Gastroenterol Clin Biol* 2009; **33** Suppl 3: S137-S144 [PMID: 20117337 DOI: 10.1016/S0399-8320(09)73149-5]
62 **Bevins CL**, Stange EF, Wehkamp J. Decreased Paneth cell defensin expression in ileal Crohn's disease is independent of inflammation, but linked to the NOD2 1007fs genotype. *Gut* 2009; **58**: 882-883; discussion 882-883 [PMID: 19433600]
63 **Wehkamp J**, Koslowski M, Wang G, Stange EF. Barrier dysfunction due to distinct defensin deficiencies in small intestinal and colonic Crohn's disease. *Mucosal Immunol* 2008; **1** Suppl 1: S67-S74 [PMID: 19079235 DOI: 10.1038/mi.2008.48]
64 **Wehkamp J**, Stange EF. A new look at Crohn's disease: breakdown of the mucosal antibacterial defense. *Ann N Y Acad Sci* 2006; **1072**: 321-331 [PMID: 17057212]
65 **Rosenberg L**, Lawlor GO, Zenlea T, Goldsmith JD, Gifford A, Falchuk KR, Wolf JL, Cheifetz AS, Robson SC, Moss AC. Predictors of endoscopic inflammation in patients with ulcerative colitis in clinical remission. *Inflamm Bowel Dis* 2013; **19**: 779-784 [PMID: 23446338 DOI: 10.1097/MIB.0b013e3182802b0e]
66 **Rosenberg L**, Nanda KS, Zenlea T, Gifford A, Lawlor GO, Falchuk KR, Wolf JL, Cheifetz AS, Goldsmith JD, Moss AC. Histologic markers of inflammation in patients with ulcerative colitis in clinical remission. *Clin Gastroenterol Hepatol* 2013; **11**: 991-996 [PMID: 23591275]
67 **D'Incà R**, Sturniolo GC, Martines D, Di Leo V, Cecchetto

A, Venturi C, Naccarato R. Functional and morphological changes in small bowel of Crohn's disease patients. Influence of site of disease. *Dig Dis Sci* 1995; **40**: 1388-1393 [PMID: 7781465]
68 **Riis P**. Inflammation as a diagnostic keystone and its clinical implications, exemplified by the inflammatory bowel diseases. *Agents Actions* 1990; **29**: 4-7 [PMID: 2183579]
69 **Tahara T**, Shibata T, Nakamura M, Okubo M, Yamashita H, Yoshioka D, Yonemura J, Kmiya Y, Ishizuka T, Fujita H, Nagasaka M, Yamada H, Hirata I, Arisawa T. Host genetic factors, related to inflammatory response, influence the CpG island methylation status in colonic mucosa in ulcerative colitis. *Anticancer Res* 2011; **31**: 933-938 [PMID: 21498716]
70 **Adamik J**, Henkel M, Ray A, Auron PE, Duerr R, Barrie A. The IL17A and IL17F loci have divergent histone modifications and are differentially regulated by prostaglandin E2 in Th17 cells. *Cytokine* 2013; **64**: 404-412 [PMID: 23800789]
71 **Ventham NT**, Kennedy NA, Nimmo ER, Satsangi J. Beyond gene discovery in inflammatory bowel disease: the emerging role of epigenetics. *Gastroenterology* 2013; **145**: 293-308 [PMID: 23751777]
72 **Stylianou E**. Epigenetics: the fine-tuner in inflammatory bowel disease? *Curr Opin Gastroenterol* 2013; **29**: 370-377 [PMID: 23743674 DOI: 10.1097/MOG.0b013e328360bd12]
73 **Jenke AC**, Zilbauer M. Epigenetics in inflammatory bowel disease. *Curr Opin Gastroenterol* 2012; **28**: 577-584 [PMID: 23041674 DOI: 10.1097/MOG.0b013e328357336b]
74 **Häsler R**, Feng Z, Bäckdahl L, Spehlmann ME, Franke A, Teschendorff A, Rakyan VK, Down TA, Wilson GA, Feber A, Beck S, Schreiber S, Rosenstiel P. A functional methylome map of ulcerative colitis. *Genome Res* 2012; **22**: 2130-2137 [PMID: 22826509 DOI: 10.1101/gr.138347.112]
75 **Cooke J**, Zhang H, Greger L, Silva AL, Massey D, Dawson C, Metz A, Ibrahim A, Parkes M. Mucosal genome-wide methylation changes in inflammatory bowel disease. *Inflamm Bowel Dis* 2012; **18**: 2128-2137 [PMID: 22419656 DOI: 10.1002/ibd.22942]
76 **Archanioti P**, Gazouli M, Theodoropoulos G, Vaiopoulou A, Nikiteas N. Micro-RNAs as regulators and possible diagnostic bio-markers in inflammatory bowel disease. *J Crohns Colitis* 2011; **5**: 520-524 [PMID: 22115369 DOI: 10.1016/j.crohns.2011.05.007]
77 **Rutgeerts P**, Diamond RH, Bala M, Olson A, Lichtenstein GR, Bao W, Patel K, Wolf DC, Safdi M, Colombel JF, Lashner B, Hanauer SB. Scheduled maintenance treatment with infliximab is superior to episodic treatment for the healing of mucosal ulceration associated with Crohn's disease. *Gastrointest Endosc* 2006; **63**: 433-442; quiz 464 [PMID: 16500392]
78 **Colombel JF**, Sandborn WJ, Reinisch W, Mantzaris GJ, Kornbluth A, Rachmilewitz D, Lichtiger S, D'Haens G, Diamond RH, Broussard DL, Tang KL, van der Woude CJ, Rutgeerts P. Infliximab, azathioprine, or combination therapy for Crohn's disease. *N Engl J Med* 2010; **362**: 1383-1395 [PMID: 20393175]
79 **D'Haens G**, Baert F, van Assche G, Caenepeel P, Vergauwe P, Tuynman H, De Vos M, van Deventer S, Stitt L, Donner A, Vermeire S, Van de Mierop FJ, Coche JC, van der Woude J, Ochsenkühn T, van Bodegraven AA, Van Hootegem PP, Lambrecht GL, Mana F, Rutgeerts P, Feagan BG, Hommes D. Early combined immunosuppression or conventional management in patients with newly diagnosed Crohn's disease: an open randomised trial. *Lancet* 2008; **371**: 660-667 [PMID: 18295023 DOI: 10.1016/S0140-6736(08)60304-9]
80 **D'Haens GR**. Top-down therapy for IBD: rationale and requisite evidence. *Nat Rev Gastroenterol Hepatol* 2010; **7**: 86-92 [PMID: 20134490]
81 **Schreiber S**. Certolizumab pegol for the treatment of Crohn's disease. *Therap Adv Gastroenterol* 2011; **4**: 375-389 [PMID: 22043230 DOI: 10.1177/1756283X11413315]
82 **Sandborn WJ**. Mucosal healing with infliximab: results from the active ulcerative colitis trials. *Gastroenterol Hepatol (N Y)* 2012; **8**: 117-119 [PMID: 22485079]
83 **Rutgeerts P**, Sandborn WJ, Feagan BG, Reinisch W, Olson A, Johanns J, Travers S, Rachmilewitz D, Hanauer SB, Lichtenstein GR, de Villiers WJ, Present D, Sands BE, Colombel JF. Infliximab for induction and maintenance therapy for ulcerative colitis. *N Engl J Med* 2005; **353**: 2462-2476 [PMID: 16339095]
84 **Sandborn WJ**, Colombel JF, D'Haens G, Van Assche G, Wolf D, Kron M, Lazar A, Robinson AM, Yang M, Chao JD, Thakkar R. One-year maintenance outcomes among patients with moderately-to-severely active ulcerative colitis who responded to induction therapy with adalimumab: subgroup analyses from ULTRA 2. *Aliment Pharmacol Ther* 2013; **37**: 204-213 [PMID: 23173821 DOI: 10.1111/apt.12145]
85 **Vecchi M**, Meucci G, Gionchetti P, Beltrami M, Di Maurizio P, Beretta L, Ganio E, Usai P, Campieri M, Fornaciari G, de Franchis R. Oral versus combination mesalazine therapy in active ulcerative colitis: a double-blind, double-dummy, randomized multicentre study. *Aliment Pharmacol Ther* 2001; **15**: 251-256 [PMID: 11148445]
86 **Malchow H**, Gertz B. A new mesalazine foam enema (Claversal Foam) compared with a standard liquid enema in patients with active distal ulcerative colitis. *Aliment Pharmacol Ther* 2002; **16**: 415-423 [PMID: 11876694]

P- Reviewers: Bessissow T, HIroshi N **S- Editor:** Wen LL
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.v19.i43.7561

World J Gastroenterol 2013 November 21; 19(43): 7561-7568
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Has the risk of colorectal cancer in inflammatory bowel disease decreased?

Nynne Nyboe Andersen, Tine Jess

Nynne Nyboe Andersen, Tine Jess, Department of Epidemiology Research, Statens Serum Institut, DK-2300 Copenhagen, Denmark
Author contributions: Andersen NN collected the material and drafted the manuscript; and Jess T discussed the topic and revised the manuscript.
Correspondence to: Nynne Nyboe Andersen, MD, Department of Epidemiology Research, Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen, Denmark. nyna@ssi.dk
Telephone: +45-32-683139 Fax: +45-32-683165
Received: August 28, 2013 Revised: October 20, 2013
Accepted: November 1, 2013
Published online: November 21, 2013

Abstract

The association between inflammatory bowel disease (IBD) and colorectal cancer (CRC) has been acknowledged for almost a century and is assumedly promoted by a chronic inflammation-driven carcinogenic process in the intestine in combination with a genetic predisposition. The magnitude of the risk of CRC in IBD remains a continuing subject of debate. The early, high risk estimates for CRC in IBD were most likely overestimated due to selected patient populations originating from tertiary referral centers with a disproportional high percentage of patients with severe disease. Later population-based studies calculating risk estimates from a broad spectrum of IBD patients have found the risk to be significantly lower. At present, there is evidence that IBD patients with longstanding and extensive disease with uncontrolled inflammation are those at increased risk. Additional, other recognized risk factors include early age at onset, family history of CRC, and concomitant primary sclerosing cholangitis. A significant amount of effort is put into identifying potential preventive factors of CRC in IBD, including surveillance programs and chemopreventive agents but the individual effect of these remains uncertain. Interestingly, recent studies have reported a decline in risk of CRC over time. Surveillance programs and the new treatment strategies, particular biological treatment might be part of the reason for the observed decline in risk of CRC in IBD over time but future studies will have investigate this assumption.

Key words: Inflammatory bowel disease; Colorectal cancer; Risk; Ulcerative colitis; Crohn's disease

Core tip: The increased risk of colorectal cancer in inflammatory bowel disease is well established. But what is the true magnitude of this increased risk, does the risk of colorectal cancer differ between ulcerative colitis and Crohn's disease and what are the significant risk factors? Further, recent studies have indicated that the risk of colorectal cancer in patients with inflammatory bowel disease is decreasing over time, potentially due to improved treatment options that lower the inflammatory burden. These are some of the subjects that will be elucidated and discussed in this review.

Andersen NN, Jess T. Has the risk of colorectal cancer in inflammatory bowel disease decreased? *World J Gastroenterol* 2013; 19(43): 7561-7568 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7561.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7561

INTRODUCTION

In almost a century it has been recognized that the risk of developing colorectal cancer (CRC) is increased in patients with longstanding inflammatory bowel disease (IBD), and it is estimated that one out of six deaths in ulcerative colitis (UC) patients and one out of 12 of all deaths in patients with Crohn's disease (CD)[1,2] is caused

by colorectal cancer. Together with the hereditary syndromes of familial adenomatous polyposis and hereditary non-polyposis colorectal cancer, IBD is in the top-3 high risk conditions for CRC. Both UC and CD carry an increased risk of CRC; however the risk is most extensively studied in UC. The augmented risk of CRC in IBD is assumedly promoted by a chronic, inflammation-driven carcinogenic process in the intestine in combination with a genetic predisposition[3]. The prognosis of sporadic CRC and IBD-related CRC is similar with a 5-year survival of 50%[4] whereas IBD-related CRC affect younger patients than sporadic CRC (60 years *vs* 70 years)[4,5].

In 1925, Crohn and Rosenberg[6] were the first to elucidate the relation between CRC and UC and in 1928, Bargen[7] further described 20 cases of colorectal cancer in patients with UC from the Mayo clinic in the United States. In 1971, de Dombal[8] reported a cumulative risk of CRC in a population from Leeds with extensive UC to be 5% after 10 years and as high as 41.8% after 25 years. These findings led to the suggestion of cancer prophylactic colonic surgery in UC patients with extensive disease and a disease course of more than 10 years, but this proposal has never been carried out in practice. Since then, substantial effort has been made to elucidate the supposed risk of CRC in IBD and has presented a considerable variety in risk estimates, leading to an ongoing debate concerning the true magnitude of the risk of CRC in IBD. Additionally, novel population-based studies have suggested a decline in risk of IBD-related CRC over time, potentially due to a shift in treatment strategies from the era of sulfasalazine, 5-aminosalicylic acid and corticosteroids, to the era of immunomodulators, such as thiopurines and tumor necrosis factor (TNF)-α antagonists[5,9].

RISK OF COLORECTAL CANCER IN ULCERATIVE COLITIS

A landmark meta-analysis including 116 studies published by Eaden *et al*[1] in 2001, found that the cumulative risk of CRC for UC patients was 2% at 10 years, 8% at 20 years, and 18% at 30 years. However as an important weakness of the meta-analysis the underlying studies were of very different methodology and many factors may have biased results. A main subject, primarily in early studies, has been the selective collection of IBD patients from tertiary referral centers with a high percentage of patients with disproportionately severe disease, thereby potentially introducing referral bias with an overestimation of the risk. This is in line with the findings in a Dutch study, comparing a cohort of 121 IBD patients with CRC from referral centers with a cohort of 160 IBD patients with CRC from general hospitals and confirmed that IBD patients from referral centers represent a subgroup with a more severe IBD-phenotype[10].

In order to approach a more unbiased risk estimate the use of population-based studies is essential with unselected cohorts of patients representing the complete and broad spectrum of disease. An early Swedish population-based study by Ekbom *et al*[11] including a cohort of 3117 patients with UC and followed from 1922-1983 found 91 cases of colorectal cancer, corresponding to a standardized incidence ratio (SIR) of 5.7 (95%CI: 4.6-7.0). A matched population-based cohort study by Bernstein *et al*[12] from 2000 revealed an increased risk of CRC in 2672 UC patients (RR = 2.75; 95%CI: 1.91-3.97) compared with a non-IBD population. In accordance, Söderlund *et al*[13] conducted a population-based study of 7607 IBD patients from Sweden diagnosed between 1954 and 1989 and found, for UC patients, a more than 2-fold increased risk of CRC compared to the background population (SIR = 2.7; 95%CI: 2.3-3.2). A Hungarian, population-based study by Lakatos *et al*[14] followed 723 UC patients for 8564 person-year from 1974 to 2004 and revealed a cumulative risk of CRC in UC of 0.6% after 10 years, 5.4% after 20 years and 7.5% after 30 years disease duration. Conversely, data from population-based studies originating from Scandinavia, Italy and the United States have reported lower risk estimates. A population-based study from Olmsted County, United States from 2006 found no overall increase in CRC in 378 UC patients (SIR = 1.1; 95%CI: 0.4-2.4), but in the subgroup of patients with extensive colitis the risk was increased 2-fold, although not reaching statistically significance (SIR = 2.4; 95%CI: 0.6-6.0)[15]. Winther *et al*[16] followed a population-based cohort of UC patients from Copenhagen County, for a median of 19 years and found no increased risk of CRC (standardized morbidity ratio: 1.05; 95%CI: 0.56-1.79). In accordance with this, a population-based study from Italy and a very recent population-based study from Northern Jutland, Denmark, did not find a significant increase in CRC in UC patients[17,18]. A recent meta-analysis[19], solely including population-based studies in order to approach an unbiased, general estimate of CRC risk in UC, found that an average of 1.6% of UC patients was diagnosed with CRC during 14 years of follow-up. This corresponds to a 2.4 (95%CI: 2.1-2.7) fold increased risk of CRC in UC. Looking at absolute risk the cumulative risk of CRC was 1.15% after 15 years, 1.69% after 20 years and 2.61% after 25 years disease duration. With 5 out of 8 studies originating from the Nordic countries the low risk has been suggested to be explained by high surgery rates and a high percentage of patients with proctitis in these countries, but this is not supported by the fact that the 3 non-Scandinavian studies revealed similar of even lower risk estimates than the Scandinavian studies. Beaugerie *et al*[20] recently published a prospective cohort study on risk of colorectal high-grade dysplasia and CRC among nearly 20000 patients with IBD enrolled in the French observational cohort CESAME (Cancer et Surrisque Associé aux Maladies Inflammatoires Intestinales En France) between May 2004 and June 2005. The authors found a 2-fold higher risk of CRC in IBD patients compared to the general population; a risk that was similar for both UC and CD. Sub-analyses revealed that this increased risk was driven by the relatively small percentage (14.6%) of patients with long-standing extensive colitis (>

10 years disease and > 50% of colon affected) with a SIR of 7 (95%CI: 4.4-10.5) compared with a non-significant increased risk in patients without long-standing extensive colitis (SIR = 1.1; 95%CI: 0.6-1.8). These risk estimates are higher than those originating from population-based studies and it is of importance to notice that data from the CESAME cohort arise from a selected IBD population. The difference in risk estimates from selected population *vs* unselected populations was addressed in a novel meta-analysis stratifying between study design and revealed a 4-fold greater risk of CRC in IBD patients when data originated from referral centers with selected patients compared with data from unselected patients from population-based studies[9].

RISK OF COLORECTAL CANCER IN CD

In contrast to the risk of CRC in UC patients, which has been comprehensively investigated, the risk of CRC in CD patients remains less explored. As with the risk of CRC in UC, studies on risk of intestinal cancer in CD have revealed inconsistent results with a variation in reported relative risk estimates from 0.8 to 20.0[21].

A meta-analysis from 2005 by Jess *et al*[22] exclusively including population-based studies and representing populations from North America, Scandinavia and Israel, estimated a pooled overall SIR for CRC in CD of 1.9 (95%CI: 1.4-2.5). Separate risk estimates for cancer in the colon and rectum resulted in a significant increased risk for colon cancer (SIR = 2.5; 95%CI: 1.7-3.5), whilst a slightly, non-significant increased pooled risk was estimated for rectum cancer (SIR = 1.4; 95%CI: 0.8-2.6). The risk of CRC cancer was significantly increased in CD patients with colonic involvement (SIR = 4.3; 95%CI: 2.0-9.4), non-statistically increased in patients with ileocolonic CD (SIR = 2.6; 95%CI: 0.8-8.2) and not increased in CD patients with pure ileal disease (SIR = 0.9; 95%CI: 0.2-4.1). Another meta-analysis from 2005, by Canavan *et al*[21] including both selected and unselected patient series studies, on risk of CRC in CD, reported similar results with an overall pooled RR of 2.5 (95%CI: 1.3-4.7) and only a significant increased risk for CD patients with colonic disease (RR = 4.5; 95%CI: 1.3-14.9). In subgroup analyses on site-specific CD the RR estimate increased for patients with colonic involvement whereas combined RR of CRC in CD patients with ileal disease was not increased (RR = 1.1; 95%CI: 0.8-1.5). A retrospective study by Herrinton *et al*[23] calculated risk of CRC cancer in a more recent IBD cohort from the Kaiser Permanente database from 1998 to 2010 and identified 29 incident CRC patients among persons with CD corresponding to a 1.6-fold higher risk of CRC compared with the general Kaiser Permanente population. In the up-dated meta-analysis from 2013 by Lutgens *et al*[9] the pooled risk estimate for CRC in CD was slightly increased (SIR = 1.6; 95%CI: 1.2-2.0) when data originated from population-based studies. Yet again, the risk was only increased in patients with colonic involvement, though not reaching statistical significance (pooled SIR = 2.0; 95%CI: 0.3-3.7).

RISK FACTORS

It is essential, in a clinical aspect to obtain knowledge of potential cancer predictive factors in order to identify subgroups of patients who need closer surveillance or more intense treatment. Known risk factors for CRC in IBD patients include young age at diagnosis, duration and anatomic extent of disease, family history of CRC, concurrent primary sclerosing cholangitis and persisting inflammation of the colon.

AGE AT ONSET

Young age at onset of colitis has been reported to be an independent risk factor for CRC. Interpretation of existing evidence is complicated as children tend to have more extensive and severe colitis compared with those diagnosed in adult age, and further have a potential for longer disease duration, a risk factor in itself.

The impact of early age at onset of IBD as a risk factor for CRC was assessed in a Danish cohort study by Jess *et al*[5]. They found that the risk of CRC varied markedly by age at diagnosis of UC; those diagnosed at childhood or adolescence (age 0-19 years) had the greatest risk (RR = 43.8; 95%CI: 27.2-70.7) followed by those diagnosed in young adulthood (age 20-39 years) with a RR of 2.65 (95%CI: 1.97-3.56). Those diagnosed with UC at the age period from 60-79 years had a risk of CRC that was below that of the background population (RR = 0.76; 95%CI: 0.62-0.92). However, as pointed out by the authors, the absolute risk of CRC was limited even in those diagnosed in young age[24]. Patients diagnosed with UC at age 0-19 had an absolute risk of CRC of 1.64% (95%CI: 0.25%-3.00%) after 25 years disease duration and for the group diagnosed at 20-39 years of age this risk was 0.80% (95%CI: 0.39%-1.20%). In matched controls the corresponding 25-year risk estimates were 0.05 (95%CI: 0.03%-0.07%) and 0.47% (95%CI: 0.43%-0.50%), respectively for the two age-groups. These data are supported by a population-based meta-analysis which showed that the standardized CRC incidence ratio was 4 times higher in IBD patients diagnosed at young age (< 30 years) compared with a non-significantly increased risk patients diagnosed at the age of 30 years or older[9], but the meta-analysis did not report absolute risks.

DISEASE EXTENT AND DURATION

UC patients with pancolitis are at highest risk, left-sided colitis carries a moderate risk, and patients with proctitis and protosigmoiditis are at similar risk of CRC as the background population. Ekbom *et al*[11] found that UC patients with pancolitis had a nearly 15-fold increased risk of CRC (SIR = 14.8; 95%CI: 11.4-18.9) as compared to the background population, in contrast to an increased risk of 2.8 for those with left-sided colitis and a non-significant increased risk of 1.7 for those with proctitis. A smaller risk was found in the population-based study by Söderlund *et al*[13] exploring the significance of site-

specific inflammation for both UC and CD, on risk of CRC. Within the cohort and compared with UC proctitis the risk of CRC cancer was 2.0 (95%CI: 1.3-3.0) for UC pancolitis, 1.2 (95%CI: 0.7-1.9) for left-sided UC, 0.9 for colonic CD (95%CI: 0.5-1.6) and 0.7 (95%CI: 0.4-1.1) for non-colonic CD. A risk of 5.6 (95%CI: 4.4-7.0) was found for UC pancolitis, compared to an overall risk for UC of 2.7 (95%CI: 2.3-3.2) when comparing with the general population. In accordance, the population-based meta-analysis by Jess *et al*[19] reported an overall risk of 4.8 (95%CI: 3.9-5.9) for UC patients with extensive disease. Backwash ileitis in UC, theoretically representing the greatest extent of disease due to ileal involvement, has been reported to carry an additional increased risk of CRC[25], but this has not been confirmed by others[26].

In addition to extent of disease, longer duration of disease is associated with an augmented risk of CRC in IBD. In a Danish, nationwide cohort study by Jess *et al*[5] the relative risk of CRC in IBD was low in the first years after diagnosis (except for an implausible high RR the first year after diagnosis, assumedly as a result of differential diagnostic problems or cases of coincidental detection of recent onset IBD in patients diagnosed with CRC), then progressively increasing with duration of IBD reaching the level of the non-IBD population after 8 years. After disease duration of 13 years the RR was significantly higher than the background population, reaching a level around 50% increase with longer follow-up. These results are consistent with the current surveillance guidelines defined by the American Gastroenterological Association[27] and the British Society for Gastroenterology[28] recommending initiation of surveillance after 8-10 years disease duration for CD and extensive UC and after 15-20 years for patients with left-sided UC. Nevertheless, studies have shown that up to a third of IBD patients develop CRC prior to the initial point of surveillance[5,20,29], thus questioning the efficacy of the present surveillance strategy.

PRIMARY SCLEROSING CHOLANGITIS

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease present in 3%-8% of patients with UC and 1%-3% of patients with CD[30-32], whereas 60%-80% of patients with PSC have IBD[33]. In 1992, Broomé *et al*[34] were the first to suggest that IBD patients with PCS potentially had an increased risk of CRC. A later study by the same group revealed an absolute cumulative risk of CRC in patients with UC and PSC of 9% after 10 years disease duration, 31% after 20 years and as high as 50% after 25 years; compared with 2%, 5% and 10% in patients with UC alone[35]. A meta-analysis published in 2002, including 11 studies concerning risk of CRC in patients with concomitant UC and PSC, revealed that 21% of UC-PSC patients developed CRC compared to 4% of UC patients without PSC, thus giving an odds ratio of 4.8 (95%CI: 3.6-6.4)[36]. The risk of CRC in CD patients with PSC is uncertain. A British case control study by Braden *et al*[37] studied risk of CRC in colonic CD/PSC patients and concluded that the presence of PSC did not increase the risk of CRC in patients with colonic CD. Thackeray *et al*[38] conducted a retrospective study in order to determine the time-interval between diagnosis of IBD, PSC and CRC and found that IBD-PSC patients developed cancer or dysplasia relatively soon after diagnosis of both IBD and PSC. Interestingly, patients with PSC and IBD typically have mild or asymptomatic pancolitis with prolonged remission and an inactive cause[39-41]. Further, studies have reported a more frequent location of cancer in the right colon in patients with IBD-PCS[42]. This could suggest a different pathogenesis in the subgroup of IBD patients with PSC compared to IBD patients in general, but these mechanisms remains unidentified.

Due to the high cumulative risk of CRC in IBD patients with PSC, the short time-interval between PSC diagnosis and CRC progression, and the predominately right-sided cancer location, it is recommended that patients with IBD-PSC should initiate an annual surveillance colonoscopy, rather than sigmoidoscopy, program already at time of PSC diagnosis[43].

FAMILY HISTORY OF COLORECTAL CANCER

Healthy individuals, with a family history of CRC, carry a near 2-fold increased risk of CRC. Few studies have assessed the significance of familial CRC, or IBD on risk of CRC in patients with IBD. A population-based study from Sweden found that a family history of CRC in IBD patients resulted in a doubling of the already increased risk of CRC in IBD, irrespectively of type and extent of IBD[44]. Further, sub-analyses revealed that IBD patients with a 1st-degree relative diagnosed with CRC before the age of 50 had an even higher risk (RR = 9.2; 95%CI: 3.7-23). A family history of IBD did not increase the risk of CRC.

SEVERITY OF INFLAMMATION

Under the assumption that inflammation is the promoting factor in development and progression of CRC in IBD it seems evident that the relationship between degree of inflammation and risk of CRC would be comprehensively investigated. Paradoxically, data are sparse. Rutter M *et al*[45] conducted a retrospective case-control study, using data on histological and endoscopic grade of inflammation from a well-established cancer surveillance program for patients with long-standing, extensive UC from the United Kingdom and found a highly significant correlation between severity of inflammation and the risk of CRC; both when using colonoscopic scores (OR = 2.5, $P = 0.001$) and histological scores (OR = 5.1, $P < 0.001$). These findings were replicated in another retrospective case-control study from Finland, concluding that the risk of dysplasia or CRC is strongly associated with the degree of inflammation in patients with UC[45].

IS THE RISK OF COLORECTAL CANCER DECREASING?

The management of IBD has changed markedly in the last decades[46] not only with advancement in medical treatments, *e.g.*, new biological therapies, surgical treatment options and improved diagnostic tools leading to early detection, but also with implementation of surveillance programs and awareness of the need of adherence to medication from a patient perspective. These factors could potentially reduce the long-term inflammation in IBD patients and thereby reduce the risk of CRC.

Eaden *et al*[1] reported in their meta-analysis an increase in incidence of IBD-related CRC over time from 1955 to 2001 by plotting cancer risk against the midpoint of 41 studies, but the result did not reach statistical significance (slope: 0.003, $P = 0.80$). Another meta-analysis reported a decline in risk over time, by pooling results on risk estimates classified by the publication year. They found an incidence rate of 4.29/1000 pyrs in the 1950s compared to an incidence rate of 1.09/1000 pyrs from 2000-2011[47]. Several other studies have shown a declining trend in risk over time. Söderlund *et al*[13] conducted a population-based study showing time-trends in incidence and mortality of CRC from 1960 to 2004 in 7607 Swedish IBD patients and reported adjusted relative risks of 1.7 (95%CI: 0.6-4.4) from 1960 to 1969, 1.3 (95%CI: 0.7-2.6) from 1970 to 1979, 1.2 (95%CI: 0.7-2.2) from 1980 to 1989, 1.1 (95%CI: 0.7-1.8) from 1990 to 1999, and 1 (reference) from 2000 to 2004, revealing a non-significant, declining trend. Compared to the general population the relative risk declined from a 5-fold increased risk of CRC in IBD in the 1960s to a 2-fold increased risk in the period from 2000 to 2004 (P for trend = 0.06). The risk of death from CRC decreased significantly during the same time period, both when comparing patients within the cohort and with the general population. Whether this decline in mortality is due to surveillance, better surgical management, better follow-up, or other changes is unanswered. Results from other studies are diverse. Herrinton *et al*[23] used data on IBD and CRC from the United States health insurance Kaiser Permanente database to report time-trends over a 14.5 year study period from 1998 to 2010. Results showed no time-trend with incidence rates of CRC per 100000 pyrs, varying from 87.9 in 1998-2001, to 67.0 in 2002-2006 and to 73.9 in 2007-2010 (P trend = 0.98) but one could argue that this time-interval is too short to reveal any trend over time. In contrast to the results from the United States, a nation-wide cohort study from Denmark revealed a decrease in risk of CRC in IBD over 30 years from 1979 to 2008[5]. During 178 million person-years of follow-up, relative risk estimates of CRC in IBD were calculated, adjusted by sex, age and calendar period and subdivided into three time-periods of 10 years from 1979 to 2008. Compared to the general population the overall RR of CRC in UC decreased from 1.34 (95%CI: 1.13-1.58) in 1979-1988 to 1.09 (95%CI: 0.90-1.33) in 1989-1998 and further to 0.57 (95%CI: 0.41-0.80) in 1999-2008. It has been argued that the observed decreased risk could be explained by the initiation of screening of CRC in the general population but first of all there is no systematic screening program for CRC in Denmark before year 2014 and further relative risks compared within the cohort, using the RR in the intermediate period from 1989-1999 as reference (hence enabling adjustment for shorter length of follow-up in recent cohorts) the risk was still significantly reduced (RR = 0.59; 95%CI: 0.39-0.90) in the late period from 1999-2008. When analyzing time-trends in CD no significant changes were observed. Likewise, a study on mortality within the same population revealed a decrease in mortality in UC patients from 1982 to 2010, largely due to a reduction in mortality from gastrointestinal orders and CRC[48]. In addition to the mentioned original studies, an updated meta-analysis by Lutgens *et al*[9] found a similar decreasing trend in risk of CRC in IBD over time in meta-regression analyses from 9 population-based studies, but the trend did not reach statistical significance, most likely due to a type Ⅱ error as only few studies were available for analysis. Overall, there may be a declining risk of CRC in IBD over time and the reason for this observation needs to be studied further.

CHEMOPREVENTION

Instead of focusing on early detection of neoplasia in IBD, the ideal would be to prevent neoplasia from ever developing. In light of the theory of an inflammation-driven carcinogenic process as a causative factor of IBD-related CRC, medical therapies reducing the inflammatory burden could potentially lower the risk of CRC in IBD. Hence, there has been an increasing interest in detecting chemopreventive agents that can reduce the overall risk of dysplasia and cancer and serve as a complement to current surveillance programs.

5-aminosalicylic acid (5-ASA) is the first line agent for maintenance therapy in mild to moderate UC and it has been shown in *in vitro* studies to have antineoplastic properties by inhibiting the nuclear kappa-B pathway which is involved in tumor progression[49]. However, the evidence of a potential chemoprophylactic effect of 5-ASA is contradictory. In 2005 Velayos *et al*[50] published a meta-analysis of 9 observational studies (3 cohort, 6 case-control) on effect of 5-ASA in preventing IBD related CRC. Pooled analysis revealed a protective effect of 5-ASA use on risk of IBD-related CRC with an odds ratio of 0.51 (95%CI: 0.37-0.69). Since then several case-control and population-based studies have not been able to detect any chemopreventive effect of 5-ASA[51-53]. Recently, Nguyen *et al*[54] published a meta-analysis solely including non-referral studies; thereby potentially reducing bias and presenting evidence that is more generalizable. The meta-analysis revealed a pooled adjusted OR of 0.95 (95%CI: 0.66-1.38) for CRC in patients with IBD treated with 5-ASA and based on these results the authors concluded that there does not seem to be a protective effect

of 5-ASA on risk of CRC in IBD.

In addition to 5-ASA, an increasing number of patients are treated with the thiopurine drugs, azathioprine and 6-mercaptopurine. Existing data on the potential chemopreventive effect of thiopurines in IBD are, however, conflicting. In the French CESAME clinical based cohort study the authors investigated the impact of thiopurines on risk of CRC in IBD[20]. Almost half of the 19.484 IBD patients had been exposed to thiopurines and among current users the adjusted hazard ratio for CRC was 0.57 (95%CI: 0.24-1.32) thereby revealing no significantly protective effect of thiopurine use on CRC risk in the general IBD population. However, in subanalysis confined to IBD patients with long-standing extensive colitis, current treatment with thiopurines reduced the risk of advanced colorectal neoplasia significantly (CRC and high grade dysplasia combined; HR = 0.28; 95%CI: 0.09-0.89). A Dutch cohort study by van Schaik *et al*[55] have further evaluated the effect of thiopurines on risk of CRC in an IBD cohort of 2578 IBD patients of whom 770 were exposed to thiopurines. They found that thiopurine exposure were associated with significantly decreased risk of developing advanced neoplasia (high grade dysplasia and colorectal cancer combined; adjusted HR = 0.10; 95%CI: 0.01-0.75). In a Danish nationwide population-based study by Pasternak *et al*[56] from 2013, the effect of thiopurine exposure on risk of CRC was assessed among 43969 IBD patients of whom 12% had been exposed to thiopurines. In contrast to the more selected French study, no difference in risk of CRC was observed among thiopurine exposed *vs* non-exposed patients (adjusted RR = 1.00; 95%CI: 0.61-1.63). Similar results were reported in another large population-based study from the United Kingdom[57].

Recent data from models of experimental colitis have indicated that TNF-α has a tumor promoting effect[58]. Few studies have been able to evaluate the effect of the new biological treatments, as TNF-α blockers, on risk of CRC due to the relatively short existence of these agents relative to the latency of CRC. In a Dutch nested case-control study by Baars *et al*[59] risk factors for IBD-related CRC were identified by comparing 173 cases of IBD-related CRC (collected from 1990 to 2006) with 393 non-CRC IBD controls. The authors found that use of TNF-α antagonist was a protective factor for the development of CRC (OR = 0.09; 95%CI: 0.01-0.68). In a Danish, nationwide population-based cohort study, the risk of CRC was compared between IBD patients exposed to TNF-α antagonist *vs* non-exposed and revealed no impact of TNF-α antagonist on risk of CRC (adjusted RR = 1.06; 95%CI: 0.33-3.40)[60]. The association between TNF-α antagonists and cancer is two-edged with hypotheses on both a tumor-promoting and a tumor preventing effect and future studies are necessary to clarify this aspect.

CONCLUSION

The absolute risk of CRC in IBD is limited. However, subgroups of IBD patients with severe, long-standing active disease who do not undergo colectomy and patients with PSC carry a greater risk of CRC than the background population. Overall, it seems evident that to prevent CRC from occurring in IBD, the goal is to minimize severity and extent of inflammation, whereas the methods used to do this (regular follow-up, medical treatment, surgery, and surveillance) act in common and not as single cancer-preventive factors. Whether new treatment strategies, particular biological treatment might be part of the reason for the observed decrease in risk of CRC in IBD over time needs further investigation.

REFERENCES

1 **Eaden JA**, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* 2001; **48**: 526-535 [PMID: 11247898 DOI: 10.1136/gut.48.4.526]

2 **Jess T**, Winther KV, Munkholm P, Langholz E, Binder V. Intestinal and extra-intestinal cancer in Crohn's disease: follow-up of a population-based cohort in Copenhagen County, Denmark. *Aliment Pharmacol Ther* 2004; **19**: 287-293 [PMID: 14984375 DOI: 10.1111/j.1365-2036.2004.01858.x]

3 **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]

4 **Rhodes JM**, Campbell BJ. Inflammation and colorectal cancer: IBD-associated and sporadic cancer compared. *Trends Mol Med* 2002; **8**: 10-16 [PMID: 11796261 DOI: 10.1016/S1471-4914(01)02194-3]

5 **Jess T**, Simonsen J, Jørgensen KT, Pedersen BV, Nielsen NM, Frisch M. Decreasing risk of colorectal cancer in patients with inflammatory bowel disease over 30 years. *Gastroenterology* 2012; **143**: 375-381.e1, quiz e13-14 [PMID: 22522090 DOI: 10.1053/j.gastro.2012.04.016]

6 **Crohn BB,** Rosenberg H. The sigmoidoscopic picture of chronic ulcerative colitis (non-specific). *Amer J Med Sci* 1925; **170**: 220-228 [DOI: 10.1097/00000441-192508010-00006]

7 **Bargen JA**. Chronic ulcerative colitis associated with malignant disease. 1928. *Dis Colon Rectum* 1994; **37**: 727-730 [PMID: 8026242 DOI: 10.1007/BF02054420]

8 **de Dombal FT**. Ulcerative colitis. Epidemiology and aetiology, course and prognosis. *Br Med J* 1971; **1**: 649-650 [PMID: 4926950 DOI: 10.1136/bmj.1.5750.649]

9 **Lutgens MW**, van Oijen MG, van der Heijden GJ, Vleggaar FP, Siersema PD, Oldenburg B. Declining risk of colorectal cancer in inflammatory bowel disease: an updated meta-analysis of population-based cohort studies. *Inflamm Bowel Dis* 2013; **19**: 789-799 [PMID: 23448792 DOI: 10.1097/MIB.0b013e31828029c0]

10 **Mooiweer E**, Baars JE, Lutgens MW, Vleggaar F, van Oijen M, Siersema PD, Kuipers EJ, van der Woude CJ, Oldenburg B. Disease severity does not affect the interval between IBD diagnosis and the development of CRC: results from two large, Dutch case series. *J Crohns Colitis* 2012; **6**: 435-440 [PMID: 22398065 DOI: 10.1016/j.crohns.2011.09.014]

11 **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]

12 **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)91: 4<854::AID-CNCR1073>3.0.CO;2-Z]

13 **Söderlund S**, Brandt L, Lapidus A, Karlén P, Broström O, Löfberg R, Ekbom A, Askling J. Decreasing time-trends of colorectal cancer in a large cohort of patients with in-

flammatory bowel disease. *Gastroenterology* 2009; **136**: 1561-1567; quiz 1818-1819 [PMID: 19422077 DOI: 10.1053/j.gastro.2009.01.064]
14 **Lakatos L**, Mester G, Erdelyi Z, David G, Pandur T, Balogh M, Fischer S, Vargha P, Lakatos PL. Risk factors for ulcerative colitis-associated colorectal cancer in a Hungarian cohort of patients with ulcerative colitis: results of a population-based study. *Inflamm Bowel Dis* 2006; **12**: 205-211 [PMID: 16534422 DOI: 10.1097/01.MIB.0000217770.21261.ce]
15 **Jess T**, Loftus EV, Velayos FS, Harmsen WS, Zinsmeister AR, Smyrk TC, Schleck CD, Tremaine WJ, Melton LJ, Munkholm P, Sandborn WJ. Risk of intestinal cancer in inflammatory bowel disease: a population-based study from olmsted county, Minnesota. *Gastroenterology* 2006; **130**: 1039-1046 [PMID: 16618397 DOI: 10.1053/j.gastro.2005.12.037]
16 **Winther KV**, Jess T, Langholz E, Munkholm P, Binder V. Long-term risk of cancer in ulcerative colitis: a population-based cohort study from Copenhagen County. *Clin Gastroenterol Hepatol* 2004; **2**: 1088-1095 [PMID: 15625654 DOI: 10.1016/S1542-3565(04)00543-9]
17 **Palli D**, Trallori G, Bagnoli S, Saieva C, Tarantino O, Ceroti M, d'Albasio G, Pacini F, Amorosi A, Masala G. Hodgkin's disease risk is increased in patients with ulcerative colitis. *Gastroenterology* 2000; **119**: 647-653 [PMID: 10982757 DOI: 10.1053/gast.2000.16487]
18 **Jess T,** Horváth-Puhó E, Fallingborg J, Rasmussen HH, Jacobsen BA. Cancer Risk in Inflammatory Bowel Disease According to Patient Phenotype and Treatment: A Danish Population-Based Cohort Study. *Am J Gastroenterol* 2013 Aug 27; Epub ahead of print [PMID: 23978954 DOI: 10.1038/ajg.2013.249]
19 **Jess T**, Rungoe C, Peyrin-Biroulet L. Risk of colorectal cancer in patients with ulcerative colitis: a meta-analysis of population-based cohort studies. *Clin Gastroenterol Hepatol* 2012; **10**: 639-645 [PMID: 22289873 DOI: 10.1016/j.cgh.2012.01.01]
20 **Beaugerie L**, Svrcek M, Seksik P, Bouvier AM, Simon T, Allez M, Brixi H, Gornet JM, Altwegg R, Beau P, Duclos B, Bourreille A, Faivre J, Peyrin-Biroulet L, Fléjou JF, Carrat F. Risk of colorectal high-grade dysplasia and cancer in a prospective observational cohort of patients with inflammatory bowel disease. *Gastroenterology* 2013; **145**: 166-175.e8 [PMID: 23541909 DOI: 10.1053/j.gastro.2013.03.044]
21 **Canavan C**, Abrams KR, Mayberry J. Meta-analysis: colorectal and small bowel cancer risk in patients with Crohn's disease. *Aliment Pharmacol Ther* 2006; **23**: 1097-1104 [PMID: 16611269 DOI: 10.1111/j.1365-2036.2006.02854.x]
22 **Jess T**, Gamborg M, Matzen P, Munkholm P, Sørensen TI. Increased risk of intestinal cancer in Crohn's disease: a meta-analysis of population-based cohort studies. *Am J Gastroenterol* 2005; **100**: 2724-2729 [PMID: 16393226 DOI: 10.1111/j.1572-0241.2005.00287.x]
23 **Herrinton LJ,** Liu L, Levin TR, Allison JE, Lewis JD, Velayos F. Incidence and mortality of colorectal adenocarcinoma in persons with inflammatory bowel disease from 1998 to 2010. *Gastroenterology* 2012; **143**: 382-389 [PMID: 22609382 DOI: 10.1053/j.gastro.2012.04.054]
24 **Langholz E**. Relative or relevant risk? *Gastroenterology* 2012; **143**: e20, author reply e20-e21 [PMID: 23000225 DOI: 10.1053/j.gastro.2012.09.039]
25 **Heuschen UA**, Hinz U, Allemeyer EH, Stern J, Lucas M, Autschbach F, Herfarth C, Heuschen G. Backwash ileitis is strongly associated with colorectal carcinoma in ulcerative colitis. *Gastroenterology* 2001; **120**: 841-847 [PMID: 11231938 DOI: 10.1053/gast.2001.22434]
26 **Haskell H**, Andrews CW, Reddy SI, Dendrinos K, Farraye FA, Stucchi AF, Becker JM, Odze RD. Pathologic features and clinical significance of "backwash" ileitis in ulcerative colitis. *Am J Surg Pathol* 2005; **29**: 1472-1481 [PMID: 16224214]
27 **Farraye FA**, Odze RD, Eaden J, Itzkowitz SH, McCabe RP, Dassopoulos T, Lewis JD, Ullman TA, James T, McLeod R, Burgart LJ, Allen J, Brill JV. AGA medical position statement on the diagnosis and management of colorectal neoplasia in inflammatory bowel disease. *Gastroenterology* 2010; **138**: 738-745 [PMID: 20141808 DOI: 10.1053/j.gastro.2009.12.037]
28 **Cairns SR**, Scholefield JH, Steele RJ, Dunlop MG, Thomas HJ, Evans GD, Eaden JA, Rutter MD, Atkin WP, Saunders BP, Lucassen A, Jenkins P, Fairclough PD, Woodhouse CR. Guidelines for colorectal cancer screening and surveillance in moderate and high risk groups (update from 2002). *Gut* 2010; **59**: 666-689 [PMID: 20427401 DOI: 10.1136/gut.2009.179804]
29 **Baars JE**, Kuipers EJ, van Haastert M, Nicolaï JJ, Poen AC, van der Woude CJ. Age at diagnosis of inflammatory bowel disease influences early development of colorectal cancer in inflammatory bowel disease patients: a nationwide, long-term survey. *J Gastroenterol* 2012; **47**: 1308-1322 [PMID: 22627504 DOI: 10.1007/s00535-012-0603-2]
30 **Fausa O**, Schrumpf E, Elgjo K. Relationship of inflammatory bowel disease and primary sclerosing cholangitis. *Semin Liver Dis* 1991; **11**: 31-39 [PMID: 2047887 DOI: 10.1055/s-2008-1040420]
31 **Olsson R**, Danielsson A, Järnerot G, Lindström E, Lööf L, Rolny P, Rydén BO, Tysk C, Wallerstedt S. Prevalence of primary sclerosing cholangitis in patients with ulcerative colitis. *Gastroenterology* 1991; **100**: 1319-1323 [PMID: 2013375]
32 **Rasmussen HH**, Fallingborg JF, Mortensen PB, Vyberg M, Tage-Jensen U, Rasmussen SN. Hepatobiliary dysfunction and primary sclerosing cholangitis in patients with Crohn's disease. *Scand J Gastroenterol* 1997; **32**: 604-610 [PMID: 9200295 DOI: 10.3109/00365529709025107]
33 **Molodecky NA**, Kareemi H, Parab R, Barkema HW, Quan H, Myers RP, Kaplan GG. Incidence of primary sclerosing cholangitis: a systematic review and meta-analysis. *Hepatology* 2011; **53**: 1590-1599 [PMID: 21351115 DOI: 10.1002/hep.24247]
34 **Broomé U**, Lindberg G, Löfberg R. Primary sclerosing cholangitis in ulcerative colitis--a risk factor for the development of dysplasia and DNA aneuploidy? *Gastroenterology* 1992; **102**: 1877-1880 [PMID: 1587406]
35 **Broomé U**, Löfberg R, Veress B, Eriksson LS. Primary sclerosing cholangitis and ulcerative colitis: evidence for increased neoplastic potential. *Hepatology* 1995; **22**: 1404-1408 [PMID: 7590655]
36 **Soetikno RM**, Lin OS, Heidenreich PA, Young HS, Blackstone MO. Increased risk of colorectal neoplasia in patients with primary sclerosing cholangitis and ulcerative colitis: a meta-analysis. *Gastrointest Endosc* 2002; **56**: 48-54 [PMID: 12085034 DOI: 10.1067/mge.2002.125367]
37 **Braden B**, Halliday J, Aryasingha S, Sharifi Y, Checchin D, Warren BF, Kitiyakara T, Travis SP, Chapman RW. Risk for colorectal neoplasia in patients with colonic Crohn's disease and concomitant primary sclerosing cholangitis. *Clin Gastroenterol Hepatol* 2012; **10**: 303-308 [PMID: 22037429 DOI: 10.1016/j.cgh.2011.10.020]
38 **Thackeray EW**, Charatcharoenwitthaya P, Elfaki D, Sinakos E, Lindor KD. Colon neoplasms develop early in the course of inflammatory bowel disease and primary sclerosing cholangitis. *Clin Gastroenterol Hepatol* 2011; **9**: 52-56 [PMID: 20920596 DOI: 10.1016/j.cgh.2010.09.020]
39 **Marchesa P**, Lashner BA, Lavery IC, Milsom J, Hull TL, Strong SA, Church JM, Navarro G, Fazio VW. The risk of cancer and dysplasia among ulcerative colitis patients with primary sclerosing cholangitis. *Am J Gastroenterol* 1997; **92**: 1285-1288 [PMID: 9260790]
40 **Shetty K**, Rybicki L, Brzezinski A, Carey WD, Lashner BA. The risk for cancer or dysplasia in ulcerative colitis patients with primary sclerosing cholangitis. *Am J Gastroenterol* 1999; **94**: 1643-1649 [PMID: 10364038]
41 **Sokol H**, Cosnes J, Chazouilleres O, Beaugerie L, Tiret E, Poupon R, Seksik P. Disease activity and cancer risk in inflammatory bowel disease associated with primary scle-

rosing cholangitis. *World J Gastroenterol* 2008; **14**: 3497-3503 [PMID: 18567077 DOI: 10.3748/wjg.14.3497]
42 **Claessen MM**, Lutgens MW, van Buuren HR, Oldenburg B, Stokkers PC, van der Woude CJ, Hommes DW, de Jong DJ, Dijkstra G, van Bodegraven AA, Siersema PD, Vleggaar FP. More right-sided IBD-associated colorectal cancer in patients with primary sclerosing cholangitis. *Inflamm Bowel Dis* 2009; **15**: 1331-1336 [PMID: 19229982 DOI: 10.1002/ibd.20886]
43 **Torres J**, Pineton de Chambrun G, Itzkowitz S, Sachar DB, Colombel JF. Review article: colorectal neoplasia in patients with primary sclerosing cholangitis and inflammatory bowel disease. *Aliment Pharmacol Ther* 2011; **34**: 497-508 [PMID: 21692821 DOI: 10.1111/j.1365-2036.2011.04753.x]
44 **Askling J**, Dickman PW, Karlén P, Broström O, Lapidus A, Löfberg R, Ekbom A. Family history as a risk factor for colorectal cancer in inflammatory bowel disease. *Gastroenterology* 2001; **120**: 1356-1362 [PMID: 11313305 DOI: 10.1053/gast.2001.24052]
45 **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 DOI: 10.1053/j.gastro.2003.11.010]
46 **Herrinton LJ**, Liu L, Fireman B, Lewis JD, Allison JE, Flowers N, Hutfless S, Velayos FS, Abramson O, Altschuler A, Perry GS. Time trends in therapies and outcomes for adult inflammatory bowel disease, Northern California, 1998-2005. *Gastroenterology* 2009; **137**: 502-511 [PMID: 19445944 DOI: 10.1053/j.gastro.2009.04.063]
47 **Castaño-Milla C**, Chaparro M, Gisbert JP. Has the risk of developing colorectal cancer in patients with ulcerative colitis been overstated? A meta-analysis. *Gastroenterology* 2013; **142**: s-251[abstract] Available from: URL: http://www.gastrojournal.org/article/S0016-5085%2812%2960944-5/abstract
48 **Jess T**, Frisch M, Simonsen J. Trends in overall and cause-specific mortality among patients with inflammatory bowel disease from 1982 to 2010. *Clin Gastroenterol Hepatol* 2013; **11**: 43-48 [PMID: 23022699 DOI: 10.1016/j.cgh.2012.09.026]
49 **Kaiser GC**, Yan F, Polk DB. Mesalamine blocks tumor necrosis factor growth inhibition and nuclear factor kappaB activation in mouse colonocytes. *Gastroenterology* 1999; **116**: 602-609 [PMID: 10029619]
50 **Velayos FS**, Terdiman JP, Walsh JM. Effect of 5-aminosalicylate use on colorectal cancer and dysplasia risk: a systematic review and metaanalysis of observational studies. *Am J Gastroenterol* 2005; **100**: 1345-1353 [PMID: 15929768 DOI: 10.1111/j.1572-0241.2005.41442.x]
51 **Bernstein CN**, Nugent Z, Blanchard JF. 5-aminosalicylate is not chemoprophylactic for colorectal cancer in IBD: a population based study. *Am J Gastroenterol* 2011; **106**: 731-736 [PMID: 21407180 DOI: 10.1038/ajg.2011.50]
52 **Jess T**, Loftus EV, Velayos FS, Winther KV, Tremaine WJ, Zinsmeister AR, Scott Harmsen W, Langholz E, Binder V, Munkholm P, Sandborn WJ. Risk factors for colorectal neoplasia in inflammatory bowel disease: a nested case-control study from Copenhagen county, Denmark and Olmsted county, Minnesota. *Am J Gastroenterol* 2007; **102**: 829-836 [PMID: 17222314 DOI: 10.1111/j.1572-0241.2007.01070.x]
53 **Terdiman JP**, Steinbuch M, Blumentals WA, Ullman TA, Rubin DT. 5-Aminosalicylic acid therapy and the risk of colorectal cancer among patients with inflammatory bowel disease. *Inflamm Bowel Dis* 2007; **13**: 367-371 [PMID: 17206695 DOI: 10.1002/ibd.20074]
54 **Nguyen GC**, Gulamhusein A, Bernstein CN. 5-aminosalicylic acid is not protective against colorectal cancer in inflammatory bowel disease: a meta-analysis of non-referral populations. *Am J Gastroenterol* 2012; **107**: 1298-304; quiz 1297, 1305 [PMID: 22751467 DOI: 10.1038/ajg.2012.198]
55 **van Schaik FD**, van Oijen MG, Smeets HM, van der Heijden GJ, Siersema PD, Oldenburg B. Thiopurines prevent advanced colorectal neoplasia in patients with inflammatory bowel disease. *Gut* 2012; **61**: 235-240 [PMID: 21602529 DOI: 10.1136/gut.2011.237412]
56 **Pasternak B**, Svanström H, Schmiegelow K, Jess T, Hviid A. Use of azathioprine and the risk of cancer in inflammatory bowel disease. *Am J Epidemiol* 2013; **177**: 1296-1305 [PMID: 23514635 DOI: 10.1093/aje/kws375]
57 **Armstrong RG**, West J, Card TR. Risk of cancer in inflammatory bowel disease treated with azathioprine: a UK population-based case-control study. *Am J Gastroenterol* 2010; **105**: 1604-1609 [PMID: 20104215 DOI: 10.1038/ajg.2009.745]
58 **Onizawa M**, Nagaishi T, Kanai T, Nagano K, Oshima S, Nemoto Y, Yoshioka A, Totsuka T, Okamoto R, Nakamura T, Sakamoto N, Tsuchiya K, Aoki K, Ohya K, Yagita H, Watanabe M. Signaling pathway via TNF-alpha/NF-kappaB in intestinal epithelial cells may be directly involved in colitis-associated carcinogenesis. *Am J Physiol Gastrointest Liver Physiol* 2009; **296**: G850-G859 [PMID: 19179628 DOI: 10.1152/ajpgi.00071.2008]
59 **Baars JE**, Looman CW, Steyerberg EW, Beukers R, Tan AC, Weusten BL, Kuipers EJ, van der Woude CJ. The risk of inflammatory bowel disease-related colorectal carcinoma is limited: results from a nationwide nested case-control study. *Am J Gastroenterol* 2011; **106**: 319-328 [PMID: 21045815 DOI: 10.1038/ajg.2010.428]
60 **Andersen NN**, Basit S, Svanström S, Pasternak B, Caspersen S, Munkholm P, Hviis A, Jess T. Tumor necrosis factor-alpha antagonists and malignancy in inflammatory bowel disease [abstract]. *J Crohns Colitis* 2012 [DOI: 10.1016/S1873-9946(12)60451-6]

P- Reviewers: Capasso R, Gazouli M, Nielsen OH
S- Editor: Cui XM **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.v19.i43.7569

World J Gastroenterol 2013 November 21; 19(43): 7569-7576
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Autoantibodies and an immune-based rat model of inflammatory bowel disease

Hadi Esmaily, Yara Sanei, Mohammad Abdollahi

Hadi Esmaily, Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran 1985717443, Iran
Yara Sanei, Mohammad Abdollahi, Faculty of Pharmacy, Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran 1417614411, Iran
Author contributions: Esmaily H, Sanei Y and Abdollahi M contributed equally to this work.
Correspondence to: Mohammad Abdollahi, Professor, Faculty of Pharmacy, Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Poursina Street, Tehran 1417614411, Iran. mohammad@tums.ac.ir
Telephone: +98-21-66959104 Fax: +98-21-66959104
Received: June 29, 2013 Revised: September 28, 2013
Accepted: October 13, 2013
Published online: November 21, 2013

Abstract

The exact causes of inflammatory bowel disease (IBD) are not yet fully defined. From a vast body of literature, we know that the immune response has long been involved in the pathogenesis of IBD, including both ulcerative colitis and Crohn's disease. A variety of specific alterations can lead to immune activation and inflammation directed to the colon, as revealed by some animal models. Current research has focused on the role of antibodies in downstream events and mechanisms of autoimmunity and inflammation. It is not well known whether the production of antibodies is a serologic consequence of IBD, or if it is a result of barrier dysfunction induced by inflammation. Here, we present a new hypothesis to distinguish the complex links between genetic susceptibility, barrier dysfunction, commensal and pathologic microbial factors and inflammatory response (especially autoantibodies) in the pathogenesis of IBD. To ascertain the hypothesis, we developed a pilot model with the concept of the presence of antibodies against enteric bacterial antigens in IBD. Results confirmed our hypothesis. Our hypothesis suggests the possibility of subcutaneous vaccination of animals with administration of all or specific enteric bacterial antigens.

Key words: Inflammatory bowel disease rat model; Pathogenesis; Barrier dysfunction; Microbial factor

Core tip: We present a new hypothesis to distinguish the complex links between genetic susceptibility, barrier dysfunction, commensal and pathologic microbial factors and inflammatory response (especially autoantibodies) in the pathogenesis of inflammatory bowel disease (IBD). In our hypothesis, we suggest that prior activation of adaptive immunity against microbial flora antigens could initiate an IBD-like chronic inflammation if something like ethanol disturbs barrier function. If this hypothesis is supported with further experiments, it would illustrate unknown aspects of IBD pathogenesis. On this basis, we have developed a new immune-based model of IBD with the presence of antibodies against enteric bacterial antigens.

Esmaily H, Sanei Y, Abdollahi M. Autoantibodies and an immune-based rat model of inflammatory bowel disease. *World J Gastroenterol* 2013; 19(43): 7569-7576 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7569.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7569

INTRODUCTION

Etiology

Investigations have demonstrated that the pathophysiology of inflammatory bowel disease (IBD) is multifactorial, but briefly host (*e.g.*, genetics, intestinal barrier and immune system function) and exogenous factors (*e.g.*,

normal luminal flora) are two basic themes[1]. The normal intestine contains a large number of immune cells in a chronic state of so-called physiologic inflammation to control the gut and to prepare it for any immunologic response. Lack of immune responsiveness to lumen antigens may be a result of oral tolerance[2]. Multiple mechanisms are involved in the induction of oral tolerance. For instance, deletion or anergy of antigen-reactive T cells or activation of regulatory CD4 T cells suppresses gut inflammation through secretion of inhibitory cytokines such as interleukin (IL)-10 and transforming growth factor β (TGF-β). In addition, a selectively permeable barrier prevents unwanted solutes, microorganisms, and luminal antigens from confronting the immune system in the internal mucosa[2-4]. In IBD, this tolerance is altered and leads to an uncontrolled inflammation; thus, IBD is considered as a breakdown in the regulatory constraints on mucosal immune response to the microbial flora or their products within the intestine. Most of this process is mediated through components of the autoimmune response to self-antigens[5].

A variety of specific alterations can lead to immune activation and inflammation directed to the colon, as revealed in animal models demonstrating murine genetic models (transgenic models). These models showed us that deleting loci of specific cytokines (*e.g.*, IL-2, IL-10, TGF-β) or their receptors or T cell antigen recognition molecules (*e.g.*, T cell antigen receptors) or interfering with intestinal barrier integrity (*e.g.*, mucus glycoprotein, deleting N-cadherin or nuclear factor κB) leads to inflammation[4].

It has been suggested that the continuous penetration of luminal antigens and unremitting stimulation of the mucosal immune system due to an increased permeability of the intestine epithelial cells may be the primary defect in patients suffering from IBD[3]. Therefore, if we consider increased epithelial permeability as the trigger, the tragedy of IBD initiates after a disruption occurring in the mucosal integrity. Then lots of macromolecule antigens in the lumen penetrate into internal compartments of the mucosa and submucosa, and subsequently become recognized by the gut immune system. Later, interstitial macrophages and dendritic cells are locally activated and release cytokines to recruit more macrophages and monocytes from the systemic circulation[6]. In normal subjects, this acute response is subsided after controlling the invasion. In genetically susceptible subjects [defects in innate immunity response (*e.g.*, NOD2, α-defensins mutations)] or in the long-term exposure to penetrated antigens in the situation of persistent integrity perturbations, or if the invader was a specific uncontrollable pathogen (*e.g.*, *Salmonella* sp., *Shigella* sp., *Campylobacter* sp., *Clostridium difficile*), antigen presenting cells (APCs) secrete cytokines, which leads to induction of differentiation in various T-cells. In this way, the adaptive immune response is turned on[7-11]. Activation of both TH1 or TH2 leads to an inflammatory response[12]. This ignition can be turned off by the regulatory systems. Generally, recovery is achieved after repair of the first alteration in intestinal permeability.

Microbial factors

Microorganisms are a likely factor in the initiation of inflammation in IBD[13]. However, the unanswered question in this area is whether microorganisms involved in the pathogenesis of IBD are commensal flora or invasive microbial pathogens?

Normal intestinal microflora may contribute to the development of IBD in susceptible individuals. This finding has been demonstrated repeatedly in murine models of IBD[14,15]. As an example, animals which are genetically altered (*e.g.*, deficient in IL-2 and IL-10) to be susceptible to IBD do not develop the disease when raised under germ-free conditions[13]. Also, intestinal lesions in IBD typically predominate in areas of the highest bacterial exposure (*e.g.*, in distal ileum and colon with 10^{12} organisms/g).

On the other hand, a number of studies have evaluated the possible role of specific infectious agents in the pathogenesis of IBD. This role has been evaluated in two ways: the relation between specific microorganisms and IBD (*e.g.*, presence of specific antibodies in serologic findings of IBD patients[16]), and the association between some acute gastroenteritis and IBD[17].

Pathogens that could be directly responsible for initiating IBD are those that the mucosal immune system may fail to control in terms of the inflammatory response (*e.g.*, *Salmonella* sp., *Shigella* sp.). These bacteria are rich in peptides having chemotactic properties (*e.g.*, formyl-methionyl-leucyl-phenylalanine). The superantigens capable of global T-lymphocyte stimulation and subsequent inflammatory response, and those producing toxins (necrotoxins, hemolysins, and enterotoxins), cause mucosal damage[8,9,16,18]. In summary, an acute infection with specific pathogens leads to a permanent uncontrollable perturbation in intestinal integrity, even though after the acute phase there is perhaps mediation of some cytokines (*e.g.*, IFN-γ), and permeability changes across the epithelium are induced. This results in continuous exposure and stimulation of the mucosal immune system with commensal flora antigens[3,19-21].

IMMUNE REGULATION AND INFLAMMATORY CASCADE DEFECTS IN IBD

As discussed later, the mucosal immune system is normally nonresponsive to luminal contents due to oral tolerance. Once inflammation is initiated, the immune inflammatory response is propagated by T cell activation in the lamina propria. CD4 T cells are of three major types: TH1, TH2, and TH17 cells. The TH1 cells secrete predominantly IFN-γ, TNF-α, IL-2, and IL-12, which

activate cell-mediated immunity by CD8 T cells (cytotoxic) resulting in transmural granulomatous inflammation resembling CD. Meanwhile, the TH2 cells can induce B-cell differentiation and humoral immunity by secreting predominantly IL-4, IL-5, and IL-13 with superficial mucosal inflammation features resembling UC[22]. TH17 cells secrete predominantly IL-17, IL-6, and granulocyte colony-stimulating factor and seem responsible for neutrophilic recruitment[5,8,23]. After activation of these cells, they produce specific cytokines and, consequently, the epithelial barrier permeability (*e.g.*, IFN-γ) is increased. Some of these cytokines have destructive and apoptotic effects on mucosal cells, which eventually allow more antigens to pass and produce more agitation of immune cells amplifying the inflammatory cascade[3,8]. In normal situations, an activated response is subsided with regulatory T cells, including designated TH3, Tr1, and CD4, and CD25 cells[23]. Their function is blocking or down-regulating the response of TH1 and TH2 either by producing specific cytokines (IL-10 and TGF-β) or *via* cell-cell contact. There is evidence which demonstrates some defects in this regulatory system in IBD-susceptible subjects[24,25].

INTESTINAL BARRIER DYSFUNCTION

The intestine is covered by a monolayer of simple columnar and non-ciliated epithelial cells that are a type of brush border cells. These are joined together by intercellular and circumferential tight junctions to form a selectively permeable membrane. This barrier prevents unwanted solutes, microorganisms, and luminal antigens from entering the internal parts. They are also part of the immune system, acting as a first-line pathogen-recognition system because they present antigens similar to classical APC. They also express toll-like receptor (TLR) 4 and, furthermore, secrete antimicrobial peptides (*e.g.*, cryptidins and defensins)[2-4,26]. However, the epithelial barrier has some guards of the innate immune system to ensure permanent immune responsiveness (*e.g.*, DC, interstitial macrophages)[27]. If anything alters the barrier function, lots of luminal antigens could pass through the submucosal layer resulting in recruitment of neutrophils and macrophages. If these cells can control the invasion, it is not necessary to call adaptive immunity components, but if the invasion takes long then adaptive immune response component will be activated. In this process, if the regulatory systems are not able to overcome the inflammatory cascade, the secreted cytokines will deteriorate and amplify the first defect in the epithelial barrier by inducing apoptosis and necrosis in the epithelial cells. In addition, a number of studies have shown that inflammatory cytokines like TNF-κ and IFN-γ may have a role in increasing intestinal barrier permeability[3,4,8]. Some animal models of IBD have shown alterations in barrier function as the first trigger contributing to pathogenesis of IBD. Furthermore, abundant evidence indicates an increased intestinal permeability in IBD patients suggesting the permanent stimulation of the mucosal immune system as the primary defect in the pathogenesis of IBD[3,28,29].

STEPS OF AUTOIMMUNITY IN IBD

The pathogenesis of IBD and most of its extra-intestinal manifestations is immunologically mediated and appears to be mainly due to an autoimmune-related process[30,31]. As discussed, after a permanent alteration in barrier function, various antigens pass through the interstitial space which finally activates T cells. In normal subjects, the response is directed definitively against the specific epitope of antigens, but commensal organisms in the lumen have adhesive antigens (*e.g.*, flagellar antigens) which adhere to the surface proteins of mucosal cells. If there are some predisposing factors, then there is a chance for APCs to process epitopes of these antigens, with parts of the mucosal surface proteins, which activate T lymphocytes against mucosal cell surface protein[8,31]. Another scenario happens when the response to the specific epitopes of antigens is cross-reactive to autoantigens. There is evidence demonstrating relations between precise human leukocyte antigen (HLA) molecules and cross-reactive cellular antigens[32,33]. However, in the TH2-mediated immune response in UC, it is thought that perhaps development of self-reactive B cells, which are triggered to produce mucosal IgG autoantibodies, results in an inflammatory response. Meanwhile, TH1 cell-mediated immunity and auto-reactive T cells (CD4 or CD8) may be primed by microbial antigens that are cross-reactive to autoantigens[34].

A long series of studies demonstrated that IBD patients possess autoantibodies, some of which became serologic biomarkers to diagnose or distinguish subtypes of this disease, such as anti-lymphocyte, anti-goblet cell, pancreatic autoantibodies, the autoantibody against tropomyosin isoform 5 (a cytoskeletal protein found in colon epithelial cells), and antibodies against red blood cell membrane antigens that cross-react with enteropathogens such as *Campylobacter* sp.[31,34,35].

We will now discuss some of the known autoantibodies in IBD pathogenesis. There is a form of perinuclear antineutrophil cytoplasmic antibody (pANCA) which is non-reactive to myeloperoxidase. It is well defined that 60%-70% of UC patients and 5%-15% of their first-degree relatives are pANCA-positive, whereas this applies to only 2%-3% of the general population. There is a relation between positive pANCA antibody status and severity of UC disease and other complications. Interestingly, pANCA in CD is associated with colonic disease that resembles UC[31,34,35]. The definite antigens to which these antibodies are directed have not been identified, but they have cross-reactions with enteric bacterial antigens.

Other studies demonstrated the presence of another

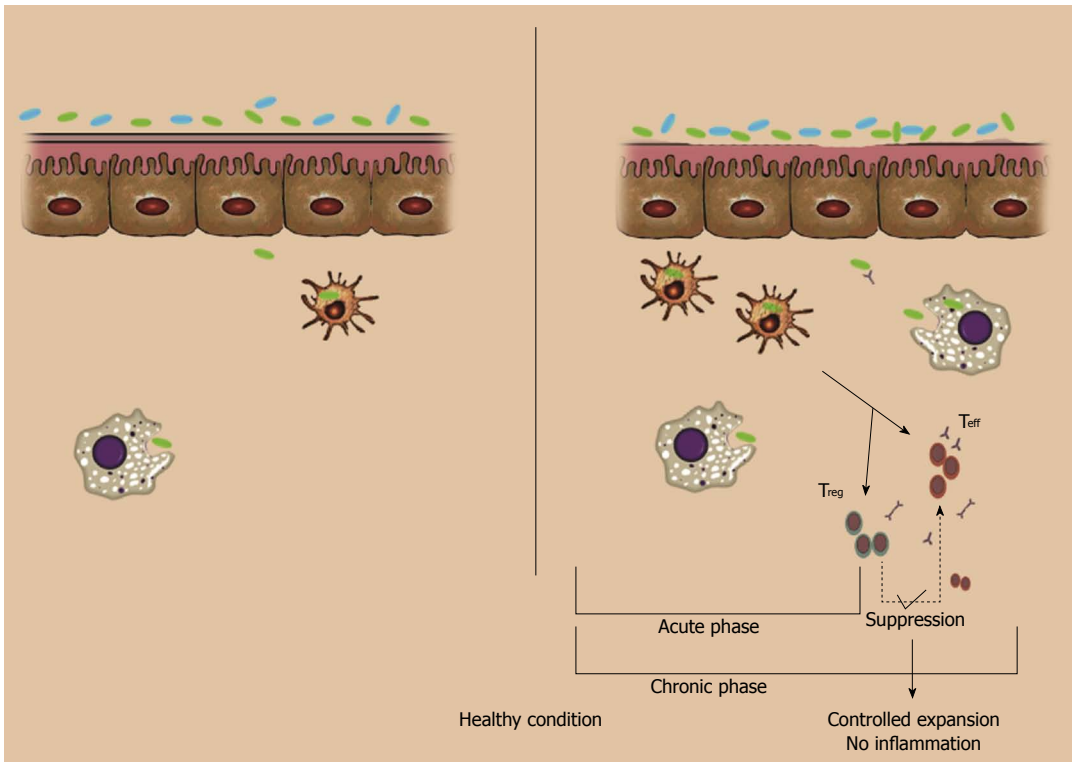


Figure 1 intestinal barrier dysfunction. Left (normal conditions): no or few commensal bacteria can pass the normal epithelial barrier and those that pass will be swallowed by interstitial macrophages and dendritic cells; it is not necessary to call for adaptive immune cells. Right: in normal situations, if something breaks the barrier (*e.g.* pathogens and barrier breaker chemicals like ethanol 30%) lots of commensal bacteria in the lumen will pass through the epithelial layer. This acute invasion will be controlled with recruiting of neutrophils and lymphocytes. Even after activation of B cells or T cells, if the defect in barrier function is resolved, apoptotic pathways will control the activated colonies of lymphocytes.

autoantibody, which is specific to patients with UC; it is an IgG autoantibody bound to a subtype of tropomyosin of colonic epithelial cell antigen. The capability of this antibody to initiate extracellular signal-regulated kinase (ERK) 1/2 signaling and up-regulating of the TLR and production of cytokines, and also the correlation between the titers of this antibody and the severity of colitis, suggest the possibility that such a protein could represent autoantigen- or complement-mediated responses[13,31].

Although the presence of antibodies directed against microbial antigens has been illustrated in the serum of CD patients, a shared epitope among the host antigens is not clearly defined. For example, 55% of CD patients have antibodies against outer membrane porin C of *Escherichia coli*, and 50% have immunoglobulins that are reactive to a homologue of the bacterial transcription-factor families from a *Pseudomonas fluorescens*-associated sequence (I_2). Around 50% of CD patients have serum reactivity to Cbir1, an immunodominant antigen of the enteric microbial flora. This antigen can strongly induce B cells and $CD4^+$ T cell responses. Transferring of Cbir1-specific $CD4^+$ TH1 T cells to C3H/SCID mice generates a severe colitis dependent on exogenous expression of Cbir1 flagellin in the colon. In 60%-70% of CD patients, anti-*Saccharomyces cerevisiae* antibodies have been found. A mannose sequence in the cell wall of this commensal flora has been defined[35,36].

HYPOTHESIS

Although the above-mentioned studies support the concept of the presence of antibodies against enteric bacterial antigens in IBD, we propose a model to investigate whether the production of antibodies is a result of barrier dysfunction induced by inflammation or a serologic finding secondary to IBD. The hypothesis would result in a reliable model of IBD studies in animals. Our hypothesis suggests the possibility of subcutaneous vaccination of animals with administration of all or specific enteric bacterial antigens. In this way, production of immunoglobulin against these antigens would prevent intestinal inflammation. Anything that alters the function of this barrier and increases barrier permeability would result in inflammatory responses. To test this hypothesis, we have designed a pilot study and examined the model in male Wistar rats, which were immunized with anaerobic and aerobic enteric bacteria with and without an adjuvant. After assessing the IgG titers in the rats' plasma, well-immunized rats were anesthetized and then chitosan and ethanol were instilled intrarectally as a tight junction opener and a barrier breaker, respectively. This protocol induced a chronic inflammatory response with inflam-matory features in the ethanol group with persistent le-

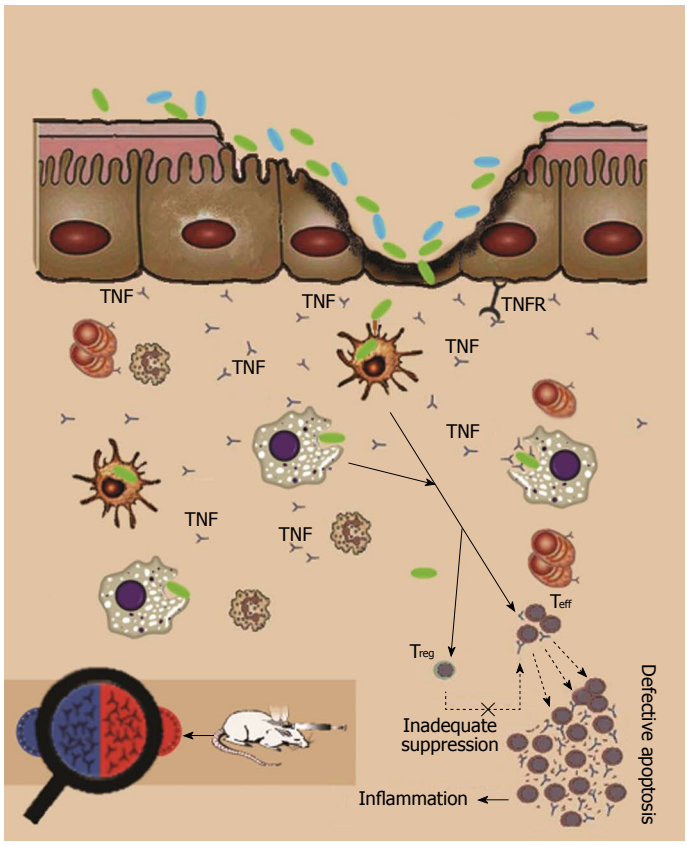


Figure 2 Steps of autoimmunity. If anything alters the barrier function, lots of luminal antigens can pass to the submucosal layer. If these cells can control the invasion, it is not necessary to call adaptive immunity components, but if the invasion take longer (*e.g.*, in altered tight junction structure) or there are some defects in innate immunity response (*e.g.*, mutations in Toll-like receptors), T cells are activated. If the regulatory system cannot overcome the inflammatory systems, cytokines and reactive destructive mediators further deteriorate the first defect *via* inducing apoptosis and necrosis in epithelial cells. Activation of T helper type 2 cells leads to a humoral response; also administration of a vaccine of commensal bacteria leads to a humoral immune response to their antigens, so after a disruption in barrier integrity with ethanol, the inflammatory cascade will turn on and induce inflammatory bowel disease in the animal as shown by histopathological findings. TNF: Tumor necrosis factor; TNFR: TNF receptor.

sions. We propose that this model of chronic intestinal inflammation would be a reliable model of human IBD. Of course, further studies would need to prove immunization with specific bacteria (Figures 1 and 2).

CONCLUSION

In this article, we addressed some known immune derangements involved in the initiation and pathogenesis of IBD. The following general principles are highlighted for better understanding of the possible mechanisms involved in the IBD pathogenesis.

Increased barrier permeability secondary to a genetic susceptibility, a specific infectious pathogen or their toxins and activation of T cells create a positive feedback to amplify the first barrier dysfunction and initiate an inflammatory cascade.

Two common features of autoimmunity processes may differ in activation of autoreactive T or B cells, involving a variety of imbalances in cytokine production and the development of autoantibodies. In IBD, these antibodies are directed against shared enteric flora antigens and epithelial cell-surface proteins.

In this study, we focused on autoantibodies. It is not well defined whether various autoantibodies found in the serologic assessment of IBD patients are destructive or involved in pathogenesis of the disease, or whether they are produced after tissue damage due to releasing of sequestered antigens[31]. We suggest that antibodies which are secreted in UC are catastrophic and are involved in the inflammatory response, but antibodies which are produced in CD are not involved in the pathogenesis and are secreted post-release of sequestered antigens. However, antibodies in both UC and CD patients are involved in extraintestinal complications, while there are various overlaps between these two subtypes.

In our hypothesis, we suggest that prior activation of adaptive immunity against microbial flora antigens in the way described could initiate an IBD-like chronic inflammation (especially in UC). Further experiments are essential to test various aspects of the method and unknown points of IBD pathogenesis.

Empirical data

After developing the hypothesis, we designed a pilot study. Six groups of male rats containing three rats in each group were considered. An extemporaneous vaccine was prepared with a mixture of heat-treated colonic commensal bacteria, which were obtained from cultured samples, and complete Freund's adjuvant. This vaccine was injected subcutaneously into nine rats on days 0 and day 14. On day 28, a blood sample was taken from each rat to assess immunoglobulin titers. All of the test animals showed an elevated titer. Then these rats were divided into three groups; intra-colonic ethanol 30% was instilled in two groups, and in the third group, normal saline was instilled instead of ethanol and this group was assigned as the vaccine group. The two groups which

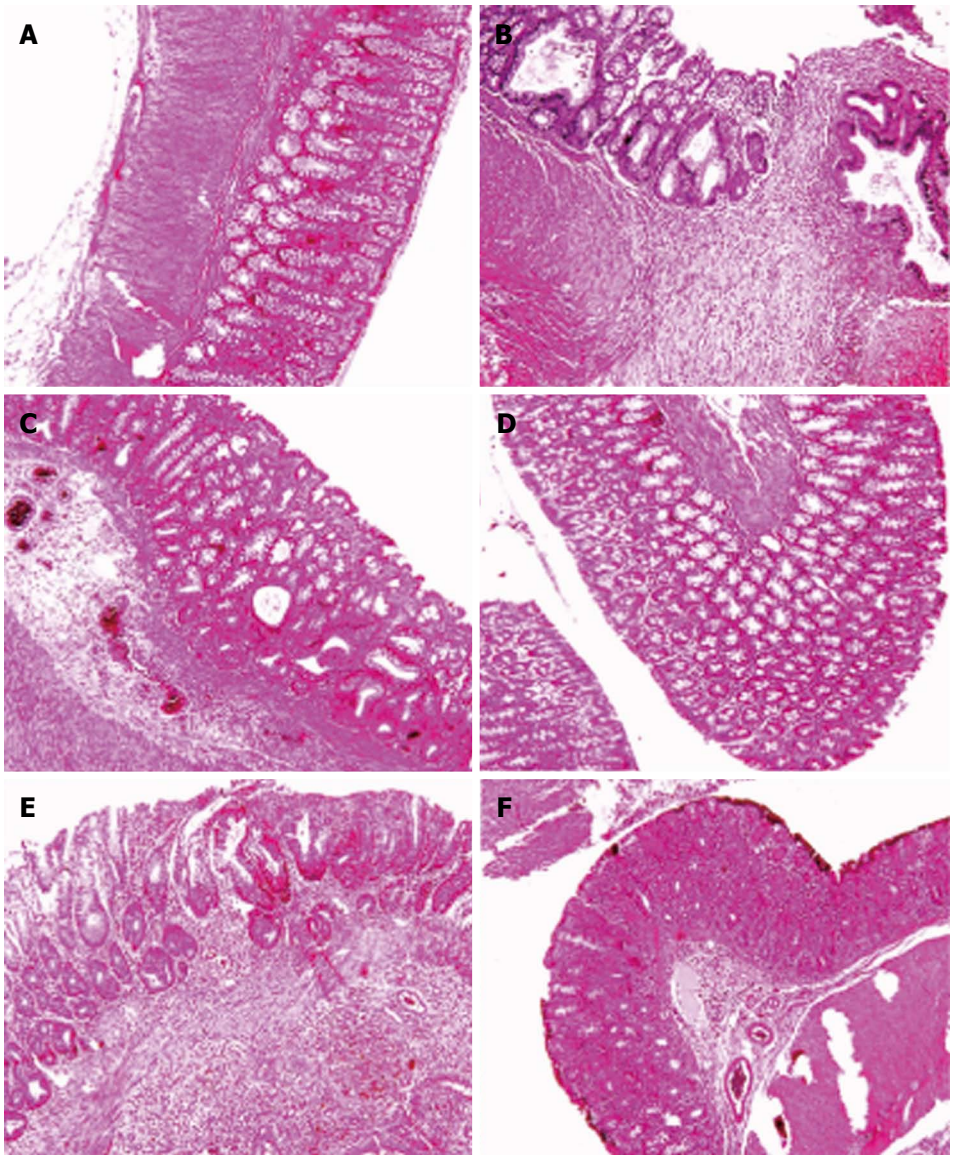


Figure 3 Histological images of colon tissues obtained from different groups. Microscopic evaluation of the trinitrobenzene sulfonic acid (TNBS) group shows villus atrophy, extensive severe transmural inflammation, granuloma with necrosis and crypt destruction, whereas features in the Sham group were normal. Histological examination of the Ethanol group showed a mild crypt distortion and some crypt abscess, whereas features in the Vaccine group were normal. Microscopic evaluation of the Model group showed mucosal inflammation and crypt distortion, branching and some ulceration with moderate to severe crypt destruction in ulcerated regions. Mild focal inflammation, minimal inflammatory cell infiltration and slight crypt branching were observed in the Infliximab group. A: Sham; B: TNBS; C: Ethanol; D: Vaccine; E: Model; F: Infliximab.

received ethanol were: the model group that received no treatment and an infliximab-treated group that received 5 mg/kg per day of infliximab for 10 consecutive days after ethanol instillation. The first of the other three groups consisted of an ethanol group that received intracolonic ethanol 30% with no pre- or post-treatment. An established colitis model was induced with instillation of 10 mg of trinitrobenzene sulfonic acid dissolved in 30% ethanol as the vehicle in another group. And the last group was normal rats (sham group), which received normal saline intracolonically. The animals were sacrificed, and colon samples were removed for histopathological assays. Details of microscopic assessments are described in Figure 3.

ACKNOWLEDGMENTS

The authors thank Tehran University of Medical Sciences for partial assistance.

REFERENCES

1 **Rezaie A**, Parker RD, Abdollahi M. Oxidative stress and pathogenesis of inflammatory bowel disease: an epiphenomenon or the cause? *Dig Dis Sci* 2007; **52**: 2015-2021 [PMID: 17404859 DOI: 10.1007/s10620-006-9622-2]
2 **Strober W**, Fuss IJ, Blumberg RS. The immunology of mucosal models of inflammation. *Annu Rev Immunol* 2002; **20**: 495-549 [PMID: 11861611 DOI: 10.1146/annurev.immunol.20.100301.064816]
3 **Laukoetter MG**, Nava P, Nusrat A. Role of the intestinal

barrier in inflammatory bowel disease. *World J Gastroenterol* 2008; **14**: 401-407 [PMID: 18200662 DOI: 10.3748/wjg.14.401]
4 **Weber CR**, Turner JR. Inflammatory bowel disease: is it really just another break in the wall? *Gut* 2007; **56**: 6-8 [PMID: 17172583 DOI: 10.1136/gut.2006.104182]
5 **Esmaily H**, Hosseini-Tabatabaei A, Rahimian R, Khorasani R, Baeeri M, Barazesh-Morgani A, Yasa N, Yassaman Khademi, Mohammad Abdollahi. On the benefits of silymarin in murine colitis by improving balance of destructive cytokines and reduction of toxic stress in the bowel cells. *Cent Eur J Biol* 2009; **4**: 204–213 [DOI: 10.2478/s11535-008-0053-2]
6 **Hosseini-Tabatabaei A**, Esmaily H, Rahimian R, Khorasani R, Baeeri M, Barazesh-Morgani A, Sari-Aslani F, Abdollahi M. Benefit of nicorandil on an immunologic murine model of experimental colitis. *Cent Eur J Biol* 2009; **4**: 74-85 [DOI: 10.2478/s11535-008-0047-0]
7 **Jurjus AR**, Khoury NN, Reimund JM. Animal models of inflammatory bowel disease. *J Pharmacol Toxicol Methods* 2004; **50**: 81-92 [PMID: 15385082 DOI: 10.1016/j.vascn.2003.12.002]
8 **Esmaily H**, Vaziri-Bami A, Miroliaee AE, Baeeri M, Abdollahi M. The correlation between NF-κB inhibition and disease activity by coadministration of silibinin and ursodeoxycholic acid in experimental colitis. *Fundam Clin Pharmacol* 2011; **25**: 723-733 [PMID: 21077947]
9 **Gradel KO**, Nielsen HL, Schønheyder HC, Ejlertsen T, Kristensen B, Nielsen H. Increased short- and long-term risk of inflammatory bowel disease after salmonella or campylobacter gastroenteritis. *Gastroenterology* 2009; **137**: 495-501 [PMID: 19361507 DOI: 10.1053/j.gastro.2009.04.001]
10 **Issa M**, Vijayapal A, Graham MB, Beaulieu DB, Otterson MF, Lundeen S, Skaros S, Weber LR, Komorowski RA, Knox JF, Emmons J, Bajaj JS, Binion DG. Impact of Clostridium difficile on inflammatory bowel disease. *Clin Gastroenterol Hepatol* 2007; **5**: 345-351 [PMID: 17368234 DOI: 10.1016/j.cgh.2006.12.028]
11 **Wen Z**, Fiocchi C. Inflammatory bowel disease: autoimmune or immune-mediated pathogenesis? *Clin Dev Immunol* 2004; **11**: 195-204 [PMID: 15559364 DOI: 10.1080/17402520400004201]
12 **Bouma G**, Strober W. The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol* 2003; **3**: 521-533 [PMID: 12876555 DOI: 10.1038/nri1132]
13 **Abdolghaffari AH**, Nikfar S, Rahimi HR, Abdollahi M. A comprehensive review of antibiotics in clinical trials for inflammatory bowel disease. *Int J Pharmacol* 2012; **8**: 596-613 [DOI: 10.3923/ijp.2012.596.613]
14 **Taurog JD**, Richardson JA, Croft JT, Simmons WA, Zhou M, Fernández-Sueiro JL, Balish E, Hammer RE. The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats. *J Exp Med* 1994; **180**: 2359-2364 [PMID: 7964509 DOI: 10.1084/jem.180.6.2359]
15 **Contractor NV**, Bassiri H, Reya T, Park AY, Baumgart DC, Wasik MA, Emerson SG, Carding SR. Lymphoid hyperplasia, autoimmunity, and compromised intestinal intraepithelial lymphocyte development in colitis-free gnotobiotic IL-2-deficient mice. *J Immunol* 1998; **160**: 385-394 [PMID: 9551995]
16 **Peyrin-Biroulet L**, Standaert-Vitse A, Branche J, Chamaillard M. IBD serological panels: facts and perspectives. *Inflamm Bowel Dis* 2007; **13**: 1561-1566 [PMID: 17636565 DOI: 10.1002/ibd.20226]
17 **Porter CK**, Tribble DR, Aliaga PA, Halvorson HA, Riddle MS. Infectious gastroenteritis and risk of developing inflammatory bowel disease. *Gastroenterology* 2008; **135**: 781-786 [PMID: 18640117 DOI: 10.1053/j.gastro.2008.05.081]
18 **Nikfar S**, Mirfazaelian H, Abdollahi M. Efficacy and tolerability of immunoregulators and antibiotics in fistulizing Crohn's disease: a systematic review and meta-analysis of placebo-controlled trials. *Curr Pharm Des* 2010; **16**: 3684-3698 [PMID: 21143147]
19 **Farrell RJ**, LaMont JT. Microbial factors in inflammatory bowel disease. *Gastroenterol Clin North Am* 2002; **31**: 41-62 [PMID: 12122743]
20 **Fasano A**, Baudry B, Pumplin DW, Wasserman SS, Tall BD, Ketley JM, Kaper JB. Vibrio cholerae produces a second enterotoxin, which affects intestinal tight junctions. *Proc Natl Acad Sci USA* 1991; **88**: 5242-5246 [PMID: 2052603 DOI: 10.1073/pnas.88.12.5242]
21 **Berkes J**, Viswanathan VK, Savkovic SD, Hecht G. Intestinal epithelial responses to enteric pathogens: effects on the tight junction barrier, ion transport, and inflammation. *Gut* 2003; **52**: 439-451 [PMID: 12584232 DOI: 10.1136/gut.52.3.439]
22 **Abdolghaffari AH**, Baghaei A, Moayer F, Esmaily H, Baeeri M, Monsef-Esfahani HR, Hajiaghaee R, Abdollahi M. On the benefit of Teucrium in murine colitis through improvement of toxic inflammatory mediators. *Hum Exp Toxicol* 2010; **29**: 287-295 [PMID: 20144954 DOI: 10.1177/0960327110361754]
23 **Sanchez-Munoz F**, Dominguez-Lopez A, Yamamoto-Furusho JK. Role of cytokines in inflammatory bowel disease. *World J Gastroenterol* 2008; **14**: 4280-4288 [PMID: 18666314 DOI: 10.3748/wjg.14.4280]
24 **Rezaie A**, Khalaj S, Shabihkhani M, Nikfar S, Zamani MJ, Mohammadirad A, Daryani NE, Abdollahi M. Study on the correlations among disease activity index and salivary transforming growth factor-beta 1 and nitric oxide in ulcerative colitis patients. *Ann N Y Acad Sci* 2007; **1095**: 305-314 [PMID: 17404043 DOI: 10.1196/annals.1397.034]
25 **Jahanshahi G**, Motavasel V, Rezaie A, Hashtroudi AA, Daryani NE, Abdollahi M. Alterations in antioxidant power and levels of epidermal growth factor and nitric oxide in saliva of patients with inflammatory bowel diseases. *Dig Dis Sci* 2004; **49**: 1752-1757 [PMID: 15628697 DOI: 10.1007/s10620-004-9564-5]
26 **Fasano A**, Shea-Donohue T. Mechanisms of disease: the role of intestinal barrier function in the pathogenesis of gastrointestinal autoimmune diseases. *Nat Clin Pract Gastroenterol Hepatol* 2005; **2**: 416-422 [PMID: 16265432 DOI: 10.1038/ncpgasthep0259]
27 **Swidsinski A**, Ladhoff A, Pernthaler A, Swidsinski S, Loening-Baucke V, Ortner M, Weber J, Hoffmann U, Schreiber S, Dietel M, Lochs H. Mucosal flora in inflammatory bowel disease. *Gastroenterology* 2002; **122**: 44-54 [PMID: 11781279 DOI: 10.1053/gast.2002.30294]
28 **Blumberg RS**, Saubermann LJ, Strober W. Animal models of mucosal inflammation and their relation to human inflammatory bowel disease. *Curr Opin Immunol* 1999; **11**: 648-656 [PMID: 10631550 DOI: 10.1016/S0952-7915(99)00032-1]
29 **Clayburgh DR**, Shen L, Turner JR. A porous defense: the leaky epithelial barrier in intestinal disease. *Lab Invest* 2004; **84**: 282-291 [PMID: 14767487 DOI: 10.1038/labinvest.3700050]
30 **Das KM**. Relationship of extraintestinal involvements in inflammatory bowel disease: new insights into autoimmune pathogenesis. *Dig Dis Sci* 1999; **44**: 1-13 [PMID: 9952216]
31 **Sandborn WJ**. Serologic markers in inflammatory bowel disease: state of the art. *Rev Gastroenterol Disord* 2004; **4**: 167-174 [PMID: 15580151]
32 **Turkcapar N**, Toruner M, Soykan I, Aydintug OT, Cetinkaya H, Duzgun N, Ozden A, Duman M. The prevalence of extraintestinal manifestations and HLA association in patients with inflammatory bowel disease. *Rheumatol Int* 2006; **26**: 663-668 [PMID: 16136311 DOI: 10.1007/s00296-005-0044-9]
33 **Orchard TR**, Chua CN, Ahmad T, Cheng H, Welsh KI, Jewell DP. Uveitis and erythema nodosum in inflammatory bowel disease: clinical features and the role of HLA genes. *Gastroenterology* 2002; **123**: 714-718 [PMID: 12198697 DOI: 10.1053/gast.2002.35396]
34 **Adams DH**, Eksteen B. Aberrant homing of mucosal T cells and extra-intestinal manifestations of inflammatory bowel

disease. *Nat Rev Immunol* 2006; **6**: 244-251 [PMID: 16498453 DOI: 10.1038/nri1784]

35 **Nakamura RM**, Matsutani M, Barry M. Advances in clinical laboratory tests for inflammatory bowel disease. *Clin Chim Acta* 2003; **335**: 9-20 [PMID: 12927679 DOI: 10.1016/S0009-8981(03)00286-9]

36 **Ferrante M**, Henckaerts L, Joossens M, Pierik M, Joossens S, Dotan N, Norman GL, Altstock RT, Van Steen K, Rutgeerts P, Van Assche G, Vermeire S. New serological markers in inflammatory bowel disease are associated with complicated disease behaviour. *Gut* 2007; **56**: 1394-1403 [PMID: 17456509 DOI: 10.1136/gut.2006.108043]

P- Reviewers: Bonaz B, Blanco Luz P, Day AS, da Silva Figueredo C, Louwen R, Ingle SB, Nayci A, Yang PC
S- Editor: Song XX **L- Editor:** Logan S **E- Editor:** Zhang DN

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v19.i43.7577

World J Gastroenterol 2013 November 21; 19(43): 7577-7585
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (3): Inflammatory bowel disease

Clostridium difficile and inflammatory bowel disease: Role in pathogenesis and implications in treatment

Orna Nitzan, Mazen Elias, Bibiana Chazan, Raul Raz, Walid Saliba

Orna Nitzan, Bibiana Chazan, Raul Raz, Infectious Disease Unit, Ha'emek Medical Center, Afula 18101, Israel
Orna Nitzan, Mazen Elias, Bibiana Chazan, Raul Raz, Walid Saliba, Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa 31096, Israel
Mazen Elias, Walid Saliba, Department of Internal Medicine C, Ha'emek Medical Center, Afula 18101, Israel
Author contributions: Nitzan O, Elias M, Chazan B, Raz R and Saliba W equally contributed to this paper.
Correspondence to: Walid Saliba, MD, MPH, Department of Internal Medicine C, Ha'emek Medical Center, 21 Rabin street, Afula 18101, Israel. saliba_wa@clalit.org.il
Telephone: +972-4-6495132 Fax: +972-4-6495134
Received: September 7, 2013 Revised: October 10, 2013
Accepted: October 13, 2013
Published online: November 21, 2013

Abstract

Clostridium difficile (*C. difficile*) is the leading cause of antibiotic associated colitis and nosocomial diarrhea. Patients with inflammatory bowel disease (IBD) are at increased risk of developing *C. difficile* infection (CDI), have worse outcomes of CDI-including higher rates of colectomy and death, and experience higher rates of recurrence. However, it is still not clear whether *C. difficile* is a cause of IBD or a consequence of the inflammatory state in the intestinal environment. The burden of CDI has increased dramatically over the past decade, with severe outbreaks described in many countries, which have been attributed to a new and more virulent strain. A parallel rise in the incidence of CDI has been noted in patients with IBD. IBD patients with CDI tend be younger, have less prior antibiotic exposure, and most cases of CDI in these patients represent outpatient acquired infections. The clinical presentation of CDI in these patients can be unique-including diversion colitis, enteritis and pouchitis, and typical findings on colonoscopy are often absent. Due to the high prevalence of CDI in patients hospitalized with an IBD exacerbation, and the prognostic implications of CDI in these patients, it is recommended to test all IBD patients hospitalized with a disease flare for *C. difficile.* Treatment includes general measures such as supportive care and infection control measures. Antibiotic therapy with either oral metronidazole, vancomycin, or the novel antibiotic-fidaxomicin, should be initiated as soon as possible. Fecal macrobiota transplantation constitutes another optional treatment for severe/recurrent CDI. The aim of this paper is to review recent data on CDI in IBD: role in pathogenesis, diagnostic methods, optional treatments, and outcomes of these patients.

Key words: *Clostridium difficile*; Diarrhea; Inflammatory bowel disease; Pathogenesis; Treatment

Core tip: In this review we focus on the role of *Clostridium difficile* (*C. difficile*) in inflammatory bowel disease pathogenesis, the unique clinical aspects of *C. difficile* infections and prognosis in patients with inflammatory bowel disease. We also present the implications of *C. difficile* infections in these patients and review the most recent literature concerning diagnostic methods and treatment.

Nitzan O, Elias M, Chazan B, Raz R, Saliba W. *Clostridium difficile* and inflammatory bowel disease: Role in pathogenesis and implications in treatment. *World J Gastroenterol* 2013; 19(43): 7577-7585 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7577.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7577

INTRODUCTION

The human gut microbiota contains about 10^{14} bacterial

cells from more than 1000 different bacterial species[1,2] that play an important role in conservation of mucosal innate and adaptive immune function, integrity of the epithelial barrier and nutrient absorption[3-6]. Disruption of the gut microbiota (dysbiosis) has been linked with many gastrointestinal conditions[7,8]. Accumulating evidence suggests that inflammatory bowel disease (IBD) results from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host[9-11]. Dysbiosis in IBD may also contribute to disease severity, and is correlated with the occurrence of abscesses in patients with Crohn's disease (CD) and need for surgery at a younger age[12,13].

Clostridium difficile (*C. difficile*) is an anaerobic gram-positive, spore-forming, toxin-producing bacillus that causes intestinal disease varying from a mild diarrheal illness to severe colitis[14-16]. The burden of *C. difficile* infection (CDI) has increased dramatically over the past decade and it is now recognized that *C. difficile* is responsible for 20%-30% of cases of antibiotic associated diarrhea and 50%-75% of cases of antibiotic associated colitis[17,18]. *C. difficile* is also the leading cause of nosocomial diarrhea, with incidence ranging from 1:100-1:1000 hospitalized patients[19,20]. Loss of intestinal microbial equilibrium, most commonly following antibiotic use, creates an environment susceptible to colonization of *C. difficile* and subsequent CDI[21,22].

IBD has been found to be associated with *C. difficile*[23-26]. Patients with IBD are at increased risk of developing CDI, have worse outcomes of CDI-including higher rates of colectomy and death, and experience higher rates of recurrence[27-30]. However, it is still not clear whether *C. difficile* is a cause of IBD or a consequence of the inflammatory state in the intestinal environment. The association between IBD and *C. difficile* may be due to different factors, such as drugs that are used for the treatment of IBD that might alter the intestinal flora and promote colonization (including repeat courses of antibiotics), altered immune and nutritional status, frequent hospitalizations, and even genetic predisposition[31,32].

In this review we will try to focus on the role of *C. difficile* in IBD pathogenesis, the unique aspects of *C. difficile* infections in patients with IBD, and the implications for testing and treatment.

The role of C. difficile in IBD

The initial trigger responsible for the onset of IBD is not yet known. A complex interplay between the immune system, environmental factors, such as stress and diet, enteric infections, and genetic factors play a role in the pathogenesis of IBD[33-35]. Gut microbiota interacts with both the innate and adaptive immune systems, playing a pivotal role in maintenance and disruption of gut immune quiescence[36]. Different bacteria have been implicated in the pathogenesis of IBD, including *Mycobacterium avium paratuberculosis,* enterotoxigenic *Bacteroides fragilis,* adherent/invasive *Escherichia coli*, *Campylobacter jejuni*, *Listeria monocytogenes*, *Chlamydia sp.*, *Aeromonas hydrophila*, *Salmonella typhi*, and *C. difficile*[37-39]. However, to date there is no conclusive evidence that a specific pathogen is responsible for IBD onset or relapse.

C. difficile has been found to be associated with IBD. Different studies found that patients with IBD, including ulcerative colitis (UC) and CD, are at increased risk of developing CDI. A study based on a large cohort of IBD patients in the United States found that CDI was more common in UC patients (2.8%) as compared to the general inpatient population (0.4%), and another study reported an adjusted odds ratios for CDI in all IBD, CD, and UC admissions from 1998-2004 to be 2.9, 4.0, and 2.1 respectively[40,41]. Since 2003 there has been a dramatic rise in the incidence of CDI with severe outbreaks described in Canada, United States and England, which have been attributed to a new and more virulent strain designated BI/NAP1/027, that has also been found in patients with IBD[18,42,43]. A parallel rise in the incidence of CDI in patients with IBD has also been noted. During 1998-2004 CDI rates approximately doubled in CD (9.5 to 22.3/1000 admissions) and tripled in UC (18.4 to 57.6/1000)[41]. A retrospective observational study found that the rate of CDI in IBD patients increased from 1.8% in 2004 to 4.6% in 2005, with the majority of patients having colonic IBD[29]. More recent studies, found that 5.5%-19% of patients with an IBD exacerbation, tested positive for *C. difficile* infection, and as many as 3.5% of children hospitalized due to IBD, were diagnosed with CDI[44,45]. Furthermore, analysis of a registry database suggests that 10% of IBD patients will develop a *C. difficile* infection at some point, and approximately 10% of CDI occur at the time of IBD diagnosis[46]. Patients with IBD also have higher rates of asymptomatic carriage of *C. difficile* 8.2% (9.4% in patients with UC and 6.9% in patients with CD), versus 1% in healthy volunteers[47]. It is possible, though, that the seemingly increased risk of CDI in patients with IBD is due to increased surveillance of this population for CDI. There are studies that question the role of CDI in IBD. A recent prospective Dutch study found a low prevalence of *C. difficile* in IBD patients and did not find any association of *C. difficile* with disease activity, disease subtype (CD or UC), gender, antibiotic, and immunosuppressive therapy[48].

Though it is still not clear if *C. difficile* causes IBD, it is understood that *C. difficile* can cause an infectious colitis superimposed on IBD, or may precipitate an IBD flare leading to simultaneous inflammatory processes, and it is nowadays considered a risk factor for IBD exacerbation. The association between *C. difficile* and IBD is mediated by a chain of events, including- recurrent hospitalizations, that are a known risk factor for acquisition of *C. difficile* and CDI, medications administered to patients with IBD (including immunomodulatory and antimicrobial agents) that disturb the intestinal flora, thus allowing for *C. difficile* colonization and adherence, and a decreased nutritional status that promotes *C. difficile* infection[31,32]. Thus, *C. difficile* can colonize the intestines of these patients and produce its two potent exotoxins: toxin A ("enterotoxin") and toxin B ("cytotoxin") that bind to receptors on intestinal epithelial cells. This activates a cascade of proinflam-

Table 1 Unique features and clinical implications of Clostridium difficile infection in patients with inflammatory bowel disease

Risk factors
Colonic IBD
Immunomodulatory drugs
In comparison to patients with no IBD: younger age, more community acquired cases, less prior antibiotic exposure
Clinical characteristics
Diarrhea (can be bloody), often mimics a flare of IBD
In patients with ileostomies: acute enteritis(an increase in ileostomy output, nausea, fever and leukocytosis)
In patients with ileal pouch anal anastomosis: pouchitis
Often no pseudomembranes on colonoscopy
Outcomes and complications
Higher rates of toxic megacolon and colonic perforation
Higher rates of colectomies
Longer length of hospital stay
Increased mortality
Diagnosis:
Test for CDI in all IBD patients hospitalized with a disease flare
As in patients with no IBD-one step molecular assays or two step algorithms: screening with EIA for GDH, followed by EIA for toxins and/or a molecular assay
Treatment:
1 Escalation of immunosuppression should be avoided during CDI
2 Antibiotics treatment as in non IBD patients
Mild to moderate disease: oral metronidazole
Severe disease: oral vancomycin + intravenous metronidazole
Fidaxomicin-less recurrences (no data in IBD patients)
3 Fecal macrobiota transplantation
Limited data in IBD patients though seems to be effective

IBD: Inflammatory bowel disease; CDI: *Clostridium difficile* infection.

matory cytokines and leukotrienes such as tumor necrosis factor (TNF), interleukin (IL)-6, IL-8, IL-1β, leukotrienes B4, and interferon-γ leading to apoptosis of gut epithelial cells and increased permeability of the intestinal mucosa, which in turn can play a role in the pathogenesis of IBD[49,50].

So, as elaborated above, there is a possible association between IBD and *C. difficile*, though there are many issues to be resolved, such as: whether CDI is a risk factor for the development of IBD, or the active inflammatory process in patients with IBD predisposes them to CDI, and what are the exact mechanisms that are responsible for this association.

CDI IN PATIENTS WITH IBD

The unique features and clinical implications of CDI in patients with IBD are summarized in table 1.

Risk factors

As noted above, patients with IBD are at increased risk for CDI, but this risk varies among different subsets of these patients. One of the major risk factors for CDI in patients with IBD is colonic IBD, either UC or CD with colitis-with 91% of patients with IBD suffering from CDI, reported to have colonic IBD, and a higher incidence of CDI was found in patients with left sided and extensive disease as compared to distal disease[29]. Other risk factors for CDI in patients with IBD are similar to those in the general population, such as- older age, medications (antibiotic/immunosuppressive agents), hospitalization, residence at a long term facility and comorbidities[21,51,52]. However, there are a few differences in risk factors for CDI in patients with IBD. IBD patients with CDI tend be younger, and 76% of *C. difficile* infections in these patients represent outpatient acquired infections, as opposed to patients without IBD were the most part of *C. difficile* infections are hospital acquired[29,53]. IBD has been found to be a risk factor for outpatient acquired CDI. In a study done in our medical center, on 115 patients with CDI, we found a trend towards a higher rate of IBD in community acquired CDI versus hospital acquired CDI (20.2% *vs* 9.7% , unpublished data).

Also, up to 40% of IBD patients do not have documented antibiotic exposure prior to presentation with CDI[54]. Patients with IBD receive various types of immunosuppressive drugs that might predispose to CDI, and steroid treatment has been found to increase the risk of CDI 3 fold in these patients, though other immunomodulatory drugs such as purine analogs, methotrexate and biological agents, have not been consistently found to increase the risk of CDI[29,41,55,56]. Combination treatment with different immunomodulatory agents can increase the risk of CDI as was found in pediatric patients receiving concomitant therapy of methotrexate and anti-TNF-α, where 28% of patients developed CDI[57].

Clinical characteristics

Patients with IBD can have different and unique clinical presentations of CDI. To begin with, the similarity in symptoms between CDI and a flare of IBD (diarrhea, abdominal pain, fever and leukocytosis) make it extremely difficult to distinguish between the two[29,41]. *C. difficile* in IBD may also show atypical features such as frequent bloody stools, as opposed to watery stools in patients without IBD. Diarrhea may even be absent in postoperative patients who receive narcotics for pain control and develop paralytic ileus. In IBD patients with ileostomies, *C. difficile* can cause acute enteritis, which can manifest as an increase in ileostomy output, nausea, fever and leukocytosis[53]. In patients who have undergone ileal pouch anal anastomosis as treatment for IBD, *C. difficile* infection might be a triggering factor for pouchitis, which presents as an increase of the number of stools per day, with or without constitutional symptoms such as weight loss[58,59]. In one study 10.7% of patients with ileal pouch anal anastomosis, presenting with pouchitis, were found to have CDI[60]. Another study demonstrated that 18.3% of cases of pouchitis were positive for *C. difficile* toxin, with men 3.5 times more likely than women to develop *C. difficile* pouchitis[61].

Typical findings of CDI on colonoscopy (such as pseudomembranous exudates, which are found in up to 60% of patients with CDI) are often absent in patients with IBD (0%-13% of cases)[62]. This might be due to a weakened inflammatory response in the colonic epithelial

environment in patients with chronic active IBD or due to immunosuppressive drugs that hamper the development of pseudomembranes, which are caused by disruption of cellular cytoskeleton by toxins, ulcer formation and leakage of serum proteins, inflammatory cells and mucus.

Outcomes

C. difficile infections have a different and often more severe clinical course in patients with IBD. These patients have higher rates of endoscopies, higher rates of complications such as toxic megacolon and colonic perforation, higher rates of colectomies, longer length of hospital stay, and increased mortality[29,30]. Different studies found high rates of colectomies in these patients, ranging from 20% to 45%[28,29], with one study finding a 6 fold increase in bowel surgery in patients with CDI with and without IBD[30]. Patients with IBD also experience more recurrences of CDI, than patients without IBD[29]. Mortality is also increased in these patients with one study demonstrating a 6%-18% case fatality rate in patients with IBD and CDI *vs* 1.4%-2.1% fatality rate in patients with CDI alone[40]. A large study of the inpatient care database in the United States found that hospitalized patients with concurrent CDI and IBD had a 4 times higher mortality rate than those admitted for IBD or CDI alone[63].

All of these special aspects of CDI in patients with IBD should cause physicians to be alert to the possibility of CDI in a patient with an IBD exacerbation and prompt rapid diagnosis and treatment.

Diagnosis

CDI is a clinical diagnosis supported by laboratory findings. As mentioned before, it is often difficult to distinguish between CDI and an exacerbation of IBD, because of the similarity in symptoms, and moreover, IBD patients may have a different clinical presentation of CDI. Laboratory findings in both CDI and IBD are also similar, including: leukocytosis, hypoalbuminemia, and fecal leukocytosis[53]. Endoscopic findings that are typical for CDI, such as colonic pseudomembranes, are also lacking in most patients with IBD that present with CDI[28]. As noted above, patients with IBD and CDI often acquire the infection in the outpatient setting and in many there is no previous documented antibiotic exposure[29,53].

Due to the high prevalence of CDI in patients hospitalized with an IBD exacerbation, the suspected causal association between CDI and flare of IBD, and the prognostic implications of CDI in these patients, it is recommended by the American college of gastroenterology CDI Guidelines Task Force, to test all IBD patients hospitalized with a disease flare for *C. difficile*[64]. Also, the European Crohn's and Colitis Organization guidelines recommend testing for Clostridium difficile infection in patients with severe or refractory UC[65]. Patients should be tested even in the absence of traditional risk factors such as antibiotic exposure.

There are various laboratory tests used in the diagnosis of CDI. Only loose, watery, or semi-formed stool should be tested for *C. difficile* and specimens should be kept at 4 ℃ if delay in testing is anticipated due to degradation of *C. difficile* toxin at room temperature[66,67]. The different tests that have been used to date are[68-70]: (1) Selective anaerobic culture: the most sensitive diagnostic method, cannot distinguish toxin-producing strains from non-toxin producing strains, time and labor consuming and thus reserved for epidemiologic studies[71]; (2) Cell culture cytotoxicity neutralization assay: detects the presence of toxin B in stool by its cytopathic effects in a cell or tissue culture, is time consuming, with a sensitivity of 65%-90%, and is rarely performed today[71]; (3) enzyme immunoassay (EIA) for *C. difficile* toxins A and B: sensitivity for toxins A and B is 60%-75% and specificity is higher (up to 99%)[72,73]. Was the routine diagnostic assay for CDI in most microbiology laboratories in recent years, but due to its low sensitivity, is not recommended today as the initial diagnostic assay[70]; (4) EIA for *C. difficile* glutamate dehydrogenase (GDH), an enzyme produced in all *C. difficile* strains: sensitivity of 75% > 90%, but cannot differentiate between toxin positive and toxin negative strains[74]; and (5) Polymerase chain reaction (PCR): detect toxin A and B genes, are highly sensitive and specific[75,76], and provide rapid results (within as little as 1 h).

Current recommendation for CDI diagnosis implement either one step molecular assays or two step algorithms with screening with EIA for GDH, followed by EIA for toxins and/or a molecular assay[67,68]. In patients with IBD, there is no evidence to date, that testing for CDI should be done differently. A recent retrospective study that compared the frequency and clinical outcomes of IBD inpatients with CDI, found that a greater percentage of patients tested positive by PCR for toxin B as compared with ELISA for toxins A + B, but the clinical outcomes were the same, regardless of method of testing[77]. More research is needed to determine the optimal diagnostic test for CDI in patients with IBD. Due to high rates of asymptomatic colonization of *C. difficile* in patients with IBD, only patients with significant diarrhea should be tested for CDI.

Treatment

Treatment of CDI includes general measures such as supportive care with attention to correction of fluid losses and electrolyte imbalances, cessation of the inciting antibiotic as soon as possible (if possible), implementation of infection control policies-including hand hygiene with soap and water which is more effective than alcohol-based hand sanitizers in eradication of *C. difficile* spores[67,78]. Antimotility agents such as loperamide and opiates have traditionally been avoided in CDI for fear of decreasing toxin clearance and increasing the risk of ileus and/or megacolon, but the evidence that they cause harm is equivocal[79].

Specific antibiotic therapy should be started as soon as possible, and empiric therapy is indicated pending results of diagnostic testing if the clinical suspicion is high and

when severe or complicated CDI is suspected. Currently, there are several drugs in use for treatment of CDI including: metronidazole (oral or intravenous), vancomycin (oral or per rectum), oral rifamixin, and a newer drug- oral fidaxomicin. A Cochrane systematic review from 2011 found no statistically significant difference in efficacy between vancomycin and other antibiotics including metronidazole, fusidic acid, nitazoxanide or rifaximin[80]. The updated guidelines for the treatment of CDI released by the Infectious Diseases Society of America and the Society for Healthcare epidemiology of America suggest that the initial choice of treatment should be determined based on the severity of illness and depending if it is a first episode of CDI or a recurrence[69]. There are different scoring systems to assess the severity of illness, including the severity score index that consists of 9 criteria, each accounting for one point: altered mental status, white blood cell count > 20000 or < 1500, albumin < 2.5 mg/dL, ascites or colitis by imaging, mean arterial pressure < 65 mmHg, fever > 38.3 ℃, tachycardia > 110 bpm and admission to intensive care unit. 1-3 points indicates mild disease, 4-6 points moderate disease, and ≥ 7 points severe disease[81]. For an initial episode of mild to moderate CDI-metronidazole, at a dose of 500 mg orally 3 times per day for 10-14 d, is considered the drug of choice. Vancomycin at a dose of 125 mg orally 4 times per day for 10-14 d is the drug of choice for an initial episode of severe CDI. Vancomycin, administered 500 mg orally 4 times per day (and 500 mg in approximately 100 mL normal saline per rectum every 6 h as a retention enema, if ileus is present) with or without intravenously administered metronidazole 500 mg intravenously every 8 h, is the regimen of choice for the treatment of severe, complicated CDI. Treatment of the first recurrence of CDI is usually with the same regimen as for the initial episode but should be stratified by disease severity, Treatment of the second or later recurrence of CDI is with vancomycin therapy using a tapered and/or pulse regimen[67].

Recent studies of fidaxomicin, 200 mg orally twice daily, compared with oral vancomycin, demonstrated non inferiority of clinical response after 10 d of treatment and superior sustained responses with a decrease in recurrences (13% *vs* 24% with vancomycin treatment)[82]. Among patients who received concomitant antibiotics, treatment with fidaxomicin resulted in higher cure rates (90% *vs* 79.4%) and lower recurrence rates (16.9% *vs* 29.2% with vancomycin)[83]. Due to these and other findings, fidaxomicin might be a promising treatment for patients with risk factors known to portend relapse and severe infection[84], though two different economical analyses reported conflicting results of the cost effectiveness of using fidaxomicin as first-line treatment for CDI[85,86].

In patients with IBD and CDI, there are no guidelines or evidence from prospective studies to suggest that one antibiotic regimen is better than another. Failure rates of up to 50% have been reported in IBD patients treated with metronidazole[87]. Considering the worse outcomes of patients with IBD and CDI, some institutions use vancomycin as first line therapy in these patients. In a single center study, where vancomycin was adopted as first line therapy in IBD patients with CDI, colectomy rates decreased from 45.5% to 25% within 1 year after the change of policy[88]. There are no data as of yet regarding the use of fidaxomicin in the IBD patient population, though in another group of immune suppressed patients-recipients of solid organ and hematopoietic stem cell transplantation, fidaxomicin achieved over all cure rates in 86% of episodes and recurrence rate was 7%[89].

Concomitant use of immunomodulators is another unresolved issue in patients with IBD and CDI. A retrospective multicenter European study comparing hospitalized IBD patients with CDI treated with antibiotics and immunomodulators or antibiotics alone, found that the primary outcome of complications including colectomy or death within 3 mo occurred in 12% of patients treated with both, as compared to none of the patients treated with antibiotics alone[90]. The use of 2 or more immunomodulators further increased the risk of complications. In a survey of North American gastroenterologists, there was significant disagreement on whether combination antibiotics and immunomodulators or antibiotics alone should be given in patients with an IBD flare and CDI[91]. The American College of Gastroenterology CDI task force, has given a conditional recommendation, with low quality supporting evidence, that ongoing immunosuppression can be maintained in patients with CDI, although escalation of immunosuppression should be avoided[64].

Fecal macrobiota transplantation (FMT) through retention enemas, rectal tube, colonoscopy, nasogastric and nasoduodenal tubes, or upper endoscopy is another option for treating recurrent CDI through restoration of a healthy microbiome in the lower gastrointestinal tract. Different studies have reported success rates of FMT approaching 90% in patients with recurrent CDI[92,93]. A randomized prospective trial, found that duodenal infusion of donor feces following vancomycin treatment was significantly more effective for the treatment of recurrent CDI than the use of vancomycin alone[94]. Data on the use of FMT among IBD patients is limited, though a recent systematic review found that out of 12 patients with IBD and CDI treated with FMT, all became toxin negative, with symptomatic resolution in 11 out of 12 patients[95]. A recent review of FMT[96] notes that though there are no guidelines concerning FMT for treatment of CDI in patients with IBD, after FMT and eradication of *c.difficile*, the severity of IBD is gradually reduced with improved responses to medications for IBD. FMT is considered a safe treatment, though a recent paper reported a case of a flare of UC in a patient who received FMT for CDI[97].

In patients after restorative proctocolectomy and ileal pouch anal anastomosis that present with *C. difficile* pouchitis, treatment is empirical because there are no published prospective trials. Studies suggest that metronidazole is not completely protective against CDI of

the pouch, as this infection has developed in patients on metronidazole therapy, thus in these patients vancomycin might be considered as first line therapy[59].

CONCLUSION

Patients with IBD are at increased risk of developing CDI and having worse outcomes, including higher rates of colectomy and death. There has also been a rise in the percentage of patients with IBD that suffer from CDI during recent years, even in those lacking classic risk factors for CDI. Patients with IBD often present with unique and more severe symptoms of CDI. Diagnosis of CDI in patients with IBD warrants a high index of suspicion and physicians should be alert to the possibility of CDI in any patient with an IBD exacerbation. All hospitalized patients with a flare of IBD should be tested for CDI and antibiotic treatment should be initiated rapidly, especially in severe cases, where vancomycin is the treatment of choice. More studies are needed to better understand the pathogenetic role of CDI in IBD exacerbations, to define what are the best diagnostic methods for CDI in these patients, to assess the efficacy of newer treatments such as fidaxomicin in patients with CDI and IBD, and to better address the question of concurrent treatment with immunomodulatory agents.

REFERENCES

1 **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 DOI: 10.1126/science.1110591]

2 **Savage DC**. Microbial ecology of the gastrointestinal tract. *Annu Rev Microbiol* 1977; **31**: 107-133 [PMID: 334036 DOI: 10.1146/annurev.mi.31.100177.000543]

3 **Zoetendal EG**, Rajilic-Stojanovic M, de Vos WM. High-throughput diversity and functionality analysis of the gastrointestinal tract microbiota. *Gut* 2008; **57**: 1605-1615 [PMID: 18941009 DOI: 10.1136/gut.2007.133603]

4 **Turnbaugh PJ**, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The human microbiome project. *Nature* 2007; **449**: 804-810 [PMID: 17943116 DOI: 10.1038/nature06244]

5 **Bäckhed F**, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-bacterial mutualism in the human intestine. *Science* 2005; **307**: 1915-1920 [PMID: 15790844 DOI: 10.1126/science.1104816]

6 **Mazmanian SK**, Liu CH, Tzianabos AO, Kasper DL. An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. *Cell* 2005; **122**: 107-118 [PMID: 16009137 DOI: 10.1016/j.cell.2005.05.007]

7 **Purchiaroni F**, Tortora A, Gabrielli M, Bertucci F, Gigante G, Ianiro G, Ojetti V, Scarpellini E, Gasbarrini A. The role of intestinal microbiota and the immune system. *Eur Rev Med Pharmacol Sci* 2013; **17**: 323-333 [PMID: 23426535]

8 **Robles Alonso V**, Guarner F. Linking the gut microbiota to human health. *Br J Nutr* 2013; **109** Suppl 2: S21-S26 [PMID: 23360877 DOI: 10.1017/S0007114512005235]

9 **Tamboli CP**, Neut C, Desreumaux P, Colombel JF. Dysbiosis in inflammatory bowel disease. *Gut* 2004; **53**: 1-4 [PMID: 14684564 DOI: 10.1136/gut.53.1.1]

10 **MacDonald TT**, Monteleone G. Overview of role of the immune system in the pathogenesis of inflammatory bowel disease. *Adv Exp Med Biol* 2006; **579**: 98-107 [PMID: 16620013 DOI: 10.1007/0-387-33778-4_6]

11 **Dupaul-Chicoine J**, Dagenais M, Saleh M. Crosstalk between the intestinal microbiota and the innate immune system in intestinal homeostasis and inflammatory bowel disease. *Inflamm Bowel Dis* 2013; **19**: 2227-2237 [PMID: 23669404 DOI: 10.1097/MIB.0b013e31828dcac7]

12 **Kaakoush NO**, Day AS, Huinao KD, Leach ST, Lemberg DA, Dowd SE, Mitchell HM. Microbial dysbiosis in pediatric patients with Crohn's disease. *J Clin Microbiol* 2012; **50**: 3258-3266 [PMID: 22837318 DOI: 10.1128/JCM.01396-12]

13 **Frank DN**, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci USA* 2007; **104**: 13780-13785 [PMID: 17699621 DOI: 10.1073/pnas.0706625104]

14 **Rubin MS**, Bodenstein LE, Kent KC. Severe Clostridium difficile colitis. *Dis Colon Rectum* 1995; **38**: 350-354 [PMID: 7720439 DOI: 10.1007/BF02054220]

15 **Lamontagne F**, Labbé AC, Haeck O, Lesur O, Lalancette M, Patino C, Leblanc M, Laverdière M, Pépin J. Impact of emergency colectomy on survival of patients with fulminant Clostridium difficile colitis during an epidemic caused by a hypervirulent strain. *Ann Surg* 2007; **245**: 267-272 [PMID: 17245181 DOI: 10.1097/01.sla.0000236628.79550.e5]

16 **Karas JA**, Enoch DA, Aliyu SH. A review of mortality due to Clostridium difficile infection. *J Infect* 2010; **61**: 1-8 [PMID: 20361997 DOI: 10.1016/j.jinf.2010.03.025]

17 **Bartlett JG**. Clostridium difficile: clinical considerations. *Rev Infect Dis* 1990; **12** Suppl 2: S243-S251 [PMID: 2406876 DOI: 10.1093/clinids/12.Supplement_2.S243]

18 **Bartlett JG**. Narrative review: the new epidemic of Clostridium difficile-associated enteric disease. *Ann Intern Med* 2006; **145**: 758-764 [PMID: 17116920 DOI: 10.7326/0003-4819-145-10-200611210-00008]

19 **Sohn S**, Climo M, Diekema D, Fraser V, Herwaldt L, Marino S, Noskin G, Perl T, Song X, Tokars J, Warren D, Wong E, Yokoe DS, Zembower T, Sepkowitz KA. Varying rates of Clostridium difficile-associated diarrhea at prevention epicenter hospitals. *Infect Control Hosp Epidemiol* 2005; **26**: 676-679 [PMID: 16156322 DOI: 10.1086/502601]

20 **McDonald LC**, Owings M, Jernigan DB. Clostridium difficile infection in patients discharged from US short-stay hospitals, 1996-2003. *Emerg Infect Dis* 2006; **12**: 409-415 [PMID: 16704777]

21 **Kelly CP**, LaMont JT. Clostridium difficile infection. *Annu Rev Med* 1998; **49**: 375-390 [PMID: 9509270 DOI: 10.1146/annurev.med.49.1.375]

22 **Thomas C**, Stevenson M, Riley TV. Antibiotics and hospital-acquired Clostridium difficile-associated diarrhoea: a systematic review. *J Antimicrob Chemother* 2003; **51**: 1339-1350 [PMID: 12746372]

23 **Bien J**, Palagani V, Bozko P. The intestinal microbiota dysbiosis and Clostridium difficile infection: is there a relationship with inflammatory bowel disease? *Therap Adv Gastroenterol* 2013; **6**: 53-68 [PMID: 23320050 DOI: 10.1177/1756283X12454590]

24 **Sinh P**, Barrett TA, Yun L. Clostridium difficile Infection and Inflammatory Bowel Disease: A Review. *Gastroenterol Res Pract* 2011; **2011**: 136064 [PMID: 21915178 DOI: 10.1155/2011/136064]

25 **Reddy SS**, Brandt LJ. Clostridium difficile infection and inflammatory bowel disease. *J Clin Gastroenterol* 2013; **47**: 666-671 [PMID: 23507767 DOI: 10.1097/MCG.0b013e31828b288a]

26 **Navaneethan U**, Venkatesh PG, Shen B. Clostridium difficile infection and inflammatory bowel disease: understanding the evolving relationship. *World J Gastroenterol* 2010; **16**: 4892-4904 [PMID: 20954275]

27 **Navaneethan U**, Mukewar S, Venkatesh PG, Lopez R, Shen B.

Clostridium difficile infection is associated with worse long term outcome in patients with ulcerative colitis. *J Crohns Colitis* 2012; **6**: 330-336 [PMID: 22405170 DOI: 10.1016/j.crohns.2011.09.005]

28 **Jodorkovsky D**, Young Y, Abreu MT. Clinical outcomes of patients with ulcerative colitis and co-existing Clostridium difficile infection. *Dig Dis Sci* 2010; **55**: 415-420 [PMID: 19255850 DOI: 10.1007/s10620-009-0749-9]

29 **Issa M**, Vijayapal A, Graham MB, Beaulieu DB, Otterson MF, Lundeen S, Skaros S, Weber LR, Komorowski RA, Knox JF, Emmons J, Bajaj JS, Binion DG. Impact of Clostridium difficile on inflammatory bowel disease. *Clin Gastroenterol Hepatol* 2007; **5**: 345-351 [PMID: 17368234]

30 **Ananthakrishnan AN**, McGinley EL, Binion DG. Excess hospitalisation burden associated with Clostridium difficile in patients with inflammatory bowel disease. *Gut* 2008; **57**: 205-210 [PMID: 17905821]

31 **Freeman HJ**. Recent developments on the role of Clostridium difficile in inflammatory bowel disease. *World J Gastroenterol* 2008; **14**: 2794-2796 [PMID: 18473400]

32 **Ananthakrishnan AN**, Oxford EC, Nguyen DD, Sauk J, Yajnik V, Xavier RJ. Genetic risk factors for Clostridium difficile infection in ulcerative colitis. *Aliment Pharmacol Ther* 2013; **38**: 522-530 [PMID: 23848254 DOI: 10.1111/apt.12425]

33 **Xavier RJ**, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 2007; **448**: 427-434 [PMID: 17653185]

34 **Cho JH**. The genetics and immunopathogenesis of inflammatory bowel disease. *Nat Rev Immunol* 2008; **8**: 458-466 [PMID: 18500230]

35 **Packey CD**, Sartor RB. Interplay of commensal and pathogenic bacteria, genetic mutations, and immunoregulatory defects in the pathogenesis of inflammatory bowel diseases. *J Intern Med* 2008; **263**: 597-606 [PMID: 18479259 DOI: 10.1111/j.1365-2796.2008.01962.x]

36 **Yu CG**, Huang Q. Recent progress on the role of gut microbiota in the pathogenesis of inflammatory bowel disease. *J Dig Dis* 2013; **14**: 513-517 [PMID: 23848393 DOI: 10.1111/1751-2980.12087]

37 **Lidar M**, Langevitz P, Shoenfeld Y. The role of infection in inflammatory bowel disease: initiation, exacerbation and protection. *Isr Med Assoc J* 2009; **11**: 558-563 [PMID: 19960852]

38 **Darfeuille-Michaud A**, Boudeau J, Bulois P, Neut C, Glasser AL, Barnich N, Bringer MA, Swidsinski A, Beaugerie L, Colombel JF. High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn's disease. *Gastroenterology* 2004; **127**: 412-421 [PMID: 15300573 DOI: 10.1053/j.gastro.2004.04.061]

39 **Burnham WR**, Lennard-Jones JE, Stanford JL, Bird RG. Mycobacteria as a possible cause of inflammatory bowel disease. *Lancet* 1978; **2**: 693-696 [PMID: 80630 DOI: 10.1016/S0140-6736(78)92699-5]

40 **Ricciardi R**, Ogilvie JW, Roberts PL, Marcello PW, Concannon TW, Baxter NN. Epidemiology of Clostridium difficile colitis in hospitalized patients with inflammatory bowel diseases. *Dis Colon Rectum* 2009; **52**: 40-45 [PMID: 19273954 DOI: 10.1007/DCR.0b013e31819733fd]

41 **Rodemann JF**, Dubberke ER, Reske KA, Seo da H, Stone CD. Incidence of Clostridium difficile infection in inflammatory bowel disease. *Clin Gastroenterol Hepatol* 2007; **5**: 339-344 [PMID: 17368233 DOI: 10.1016/j.cgh.2006.12.027]

42 **McDonald LC**, Killgore GE, Thompson A, Owens RC, Kazakova SV, Sambol SP, Johnson S, Gerding DN. An epidemic, toxin gene-variant strain of Clostridium difficile. *N Engl J Med* 2005; **353**: 2433-2441 [PMID: 16322603 DOI: 10.1056/NEJMoa051590]

43 **Goorhuis A**, Van der Kooi T, Vaessen N, Dekker FW, Van den Berg R, Harmanus C, van den Hof S, Notermans DW, Kuijper EJ. Spread and epidemiology of Clostridium difficile polymerase chain reaction ribotype 027/toxinotype III in The Netherlands. *Clin Infect Dis* 2007; **45**: 695-703 [PMID: 17712752 DOI: 10.1086/520984]

44 **Meyer AM**, Ramzan NN, Loftus EV, Heigh RI, Leighton JA. The diagnostic yield of stool pathogen studies during relapses of inflammatory bowel disease. *J Clin Gastroenterol* 2004; **38**: 772-775 [PMID: 15365403 DOI: 10.1097/01.mcg.0000139057.05297.d6]

45 **Pant C**, Anderson MP, Deshpande A, Altaf MA, Grunow JE, Atreja A, Sferra TJ. Health care burden of Clostridium difficile infection in hospitalized children with inflammatory bowel disease. *Inflamm Bowel Dis* 2013; **19**: 1080-1085 [PMID: 23478808 DOI: 10.1097/MIB.0b013e3182807563]

46 **Binion DG**. Clostridium difficile Infection in Patients with Inflammatory Bowel Disease. *Gastroenterol Hepatol* (NY) 2012; **8**: 615-617 [PMID: 23483861]

47 **Clayton EM**, Rea MC, Shanahan F, Quigley EM, Kiely B, Hill C, Ross RP. The vexed relationship between Clostridium difficile and inflammatory bowel disease: an assessment of carriage in an outpatient setting among patients in remission. *Am J Gastroenterol* 2009; **104**: 1162-1169 [PMID: 19319128 DOI: 10.1038/ajg.2009.4]

48 **Masclee GM**, Penders J, Jonkers DM, Wolffs PF, Pierik MJ. Is clostridium difficile associated with relapse of inflammatory bowel disease? results from a retrospective and prospective cohort study in the Netherlands. *Inflamm Bowel Dis* 2013; **19**: 2125-2131 [PMID: 23867869 DOI: 10.1097/MIB.0b013e318297d222]

49 **Rocha MF**, Maia ME, Bezerra LR, Lyerly DM, Guerrant RL, Ribeiro RA, Lima AA. Clostridium difficile toxin A induces the release of neutrophil chemotactic factors from rat peritoneal macrophages: role of interleukin-1beta, tumor necrosis factor alpha, and leukotrienes. *Infect Immun* 1997; **65**: 2740-2746 [PMID: 9199444]

50 **Warney M,** Kelly CP. Pathogenicity of Clostridium Difficile toxins, in Microbial Pathogenesis and the Intestinal Epithelial Cell, G. Hecht, Editors. Washington (DC): American Society for Microbiology Press, 2003: 503-524

51 **Pépin J**, Valiquette L, Cossette B. Mortality attributable to nosocomial Clostridium difficile-associated disease during an epidemic caused by a hypervirulent strain in Quebec. *CMAJ* 2005; **173**: 1037-1042 [PMID: 16179431 DOI: 10.1503/cmaj.050978]

52 **Loo VG**, Bourgault AM, Poirier L, Lamothe F, Michaud S, Turgeon N, Toye B, Beaudoin A, Frost EH, Gilca R, Brassard P, Dendukuri N, Béliveau C, Oughton M, Brukner I, Dascal A. Host and pathogen factors for Clostridium difficile infection and colonization. *N Engl J Med* 2011; **365**: 1693-1703 [PMID: 22047560 DOI: 10.1056/NEJMoa1012413]

53 **Issa M**, Ananthakrishnan AN, Binion DG. Clostridium difficile and inflammatory bowel disease. *Inflamm Bowel Dis* 2008; **14**: 1432-1442 [PMID: 18484669 DOI: 10.1002/ibd.20500]

54 **Bossuyt P**, Verhaegen J, Van Assche G, Rutgeerts P, Vermeire S. Increasing incidence of Clostridium difficile-associated diarrhea in inflammatory bowel disease. *J Crohns Colitis* 2009; **3**: 4-7 [PMID: 21172241 DOI: 10.1016/j.crohns.2008.09.003]

55 **Schneeweiss S**, Korzenik J, Solomon DH, Canning C, Lee J, Bressler B. Infliximab and other immunomodulating drugs in patients with inflammatory bowel disease and the risk of serious bacterial infections. *Aliment Pharmacol Ther* 2009; **30**: 253-264 [PMID: 19438424 DOI: 10.1111/j.1365-2036.2009.04037.x]

56 **Kariv R**, Navaneethan U, Venkatesh PG, Lopez R, Shen B. Impact of Clostridium difficile infection in patients with ulcerative colitis. *J Crohns Colitis* 2011; **5**: 34-40 [PMID: 21272802 DOI: 10.1016/j.crohns.2010.09.007]

57 **Absah I**, Faubion WA. Concomitant therapy with methotrexate and anti-TNF-α in pediatric patients with refractory crohn's colitis: a case series. *Inflamm Bowel Dis* 2012; **18**: 1488-1492 [PMID: 21882301 DOI: 10.1002/ibd.21885]

58 **Tsironi E**, Irving PM, Feakins RM, Rampton DS. "Diversion" colitis caused by Clostridium difficile infection: report of a case. *Dis Colon Rectum* 2006; **49**: 1074-1077 [PMID: 16729217]

59 **Mann SD**, Pitt J, Springall RG, Thillainayagam AV. Clostridium difficile infection--an unusual cause of refractory pouchitis: report of a case. *Dis Colon Rectum* 2003; **46**: 267-270 [PMID: 12576902]

60 **Li Y**, Qian J, Queener E, Shen B. Risk factors and outcome of PCR-detected Clostridium difficile infection in ileal pouch patients. *Inflamm Bowel Dis* 2013; **19**: 397-403 [PMID: 23328770 DOI: 10.1097/MIB.0b013e318280fcb9]

61 **Shen BO**, Jiang ZD, Fazio VW, Remzi FH, Rodriguez L, Bennett AE, Lopez R, Queener E, Dupont HL. Clostridium difficile infection in patients with ileal pouch-anal anastomosis. *Clin Gastroenterol Hepatol* 2008; **6**: 782-788 [PMID: 18467184 DOI: 10.1016/j.cgh.2008.02.021]

62 **Ben-Horin S**, Margalit M, Bossuyt P, Maul J, Shapira Y, Bojic D, Chermesh I, Al-Rifai A, Schoepfer A, Bosani M, Allez M, Lakatos PL, Bossa F, Eser A, Stefanelli T, Carbonnel F, Katsanos K, Checchin D, de Miera IS, Reinisch W, Chowers Y, Moran GW. Prevalence and clinical impact of endoscopic pseudomembranes in patients with inflammatory bowel disease and Clostridium difficile infection. *J Crohns Colitis* 2010; **4**: 194-198 [PMID: 21122505 DOI: 10.1016/j.crohns.2009.11.001]

63 **Ananthakrishnan AN**, McGinley EL, Saeian K, Binion DG. Temporal trends in disease outcomes related to Clostridium difficile infection in patients with inflammatory bowel disease. *Inflamm Bowel Dis* 2011; **17**: 976-983 [PMID: 20824818 DOI: 10.1002/ibd.21457]

64 **Surawicz CM**, Brandt LJ, Binion DG, Ananthakrishnan AN, Curry SR, Gilligan PH, McFarland LV, Mellow M, Zuckerbraun BS. Guidelines for diagnosis, treatment, and prevention of Clostridium difficile infections. *Am J Gastroenterol* 2013; **108**: 478-98; quiz 499 [PMID: 23439232 DOI: 10.1038/ajg.2013.4]

65 **Travis SP**, Stange EF, Lémann M, Oresland T, Bemelman WA, Chowers Y, Colombel JF, D'Haens G, Ghosh S, Marteau P, Kruis W, Mortensen NJ, Penninckx F, Gassull M. European evidence-based Consensus on the management of ulcerative colitis: Current management. *J Crohns Colitis* 2008; **2**: 24-62 [PMID: 21172195 DOI: 10.1016/j.crohns.2007.11.002]

66 **Gerding DN**. Diagnosis of Clostridium difficile--associated disease: patient selection and test perfection. *Am J Med* 1996; **100**: 485-486 [PMID: 8644758]

67 **Cohen SH**, Gerding DN, Johnson S, Kelly CP, Loo VG, McDonald LC, Pepin J, Wilcox MH. Clinical practice guidelines for Clostridium difficile infection in adults: 2010 update by the society for healthcare epidemiology of America (SHEA) and the infectious diseases society of America (IDSA). *Infect Control Hosp Epidemiol* 2010; **31**: 431-455 [PMID: 20307191 DOI: 10.1086/651706]

68 **Kufelnicka AM**, Kirn TJ. Effective utilization of evolving methods for the laboratory diagnosis of Clostridium difficile infection. *Clin Infect Dis* 2011; **52**: 1451-1457 [PMID: 21628487 DOI: 10.1093/cid/cir201]

69 **Burnham CA**, Carroll KC. Diagnosis of Clostridium difficile infection: an ongoing conundrum for clinicians and for clinical laboratories. *Clin Microbiol Rev* 2013; **26**: 604-630 [PMID: 23824374 DOI: 10.1128/CMR.00016-13]

70 **Katsnelson BA**, Polzik EV. Histocompatibility antigens in a population based silicosis series. *Br J Ind Med* 1990; **47**: 432 [PMID: 2378823]

71 **Shanholtzer CJ**, Willard KE, Holter JJ, Olson MM, Gerding DN, Peterson LR. Comparison of the VIDAS Clostridium difficile toxin A immunoassay with C. difficile culture and cytotoxin and latex tests. *J Clin Microbiol* 1992; **30**: 1837-1840 [PMID: 1629341]

72 **Swindells J**, Brenwald N, Reading N, Oppenheim B. Evaluation of diagnostic tests for Clostridium difficile infection. *J Clin Microbiol* 2010; **48**: 606-608 [PMID: 20032256 DOI: 10.1128/JCM.01579-09]

73 **Alcalá L**, Sánchez-Cambronero L, Catalán MP, Sánchez-Somolinos M, Peláez MT, Marín M, Bouza E. Comparison of three commercial methods for rapid detection of Clostridium difficile toxins A and B from fecal specimens. *J Clin Microbiol* 2008; **46**: 3833-3835 [PMID: 18784313 DOI: 10.1128/JCM.01060-08]

74 **Carman RJ**, Wickham KN, Chen L, Lawrence AM, Boone JH, Wilkins TD, Kerkering TM, Lyerly DM. Glutamate dehydrogenase is highly conserved among Clostridium difficile ribotypes. *J Clin Microbiol* 2012; **50**: 1425-1426 [PMID: 22301027 DOI: 10.1128/JCM.05600-11]

75 **Bélanger SD**, Boissinot M, Clairoux N, Picard FJ, Bergeron MG. Rapid detection of Clostridium difficile in feces by real-time PCR. *J Clin Microbiol* 2003; **41**: 730-734 [PMID: 12574274]

76 **Terhes G**, Urbán E, Sóki J, Nacsa E, Nagy E. Comparison of a rapid molecular method, the BD GeneOhm Cdiff assay, to the most frequently used laboratory tests for detection of toxin-producing Clostridium difficile in diarrheal feces. *J Clin Microbiol* 2009; **47**: 3478-3481 [PMID: 19794052 DOI: 10.1128/JCM.01133-09]

77 **Wang Y**, Atreja A, Wu X, Lashner BA, Brzezinski A, Shen B. Similar outcomes of IBD inpatients with Clostridium difficile infection detected by ELISA or PCR assay. *Dig Dis Sci* 2013; **58**: 2308-2313 [PMID: 23525735 DOI: 10.1007/s10620-013-2641-x]

78 **Hu MY**, Katchar K, Kyne L, Maroo S, Tummala S, Dreisbach V, Xu H, Leffler DA, Kelly CP. Prospective derivation and validation of a clinical prediction rule for recurrent Clostridium difficile infection. *Gastroenterology* 2009; **136**: 1206-1214 [PMID: 19162027 DOI: 10.1053/j.gastro.2008.12.038]

79 **Koo HL**, Koo DC, Musher DM, DuPont HL. Antimotility agents for the treatment of Clostridium difficile diarrhea and colitis. *Clin Infect Dis* 2009; **48**: 598-605 [PMID: 19191646 DOI: 10.1086/596711]

80 **Nelson RL**, Kelsey P, Leeman H, Meardon N, Patel H, Paul K, Rees R, Taylor B, Wood E, Malakun R. Antibiotic treatment for Clostridium difficile-associated diarrhea in adults. *Cochrane Database Syst Rev* 2011; (**9**): CD004610 [PMID: 21901692 DOI: 10.1002/14651858.CD004610.pub4]

81 **Toro DH,** Amaral-Mojica KM, Rocha-Rodriguez R, Gutierrez-Nunez J. An innovative Severity Score Index for Clostridium difficile Infection. A prospective study. *Infect Dis in Clin Prac* 2011; **19**: 336-339 [DOI: 10.1097IPC.0b013e31821895a8]

82 **Cornely OA**, Crook DW, Esposito R, Poirier A, Somero MS, Weiss K, Sears P, Gorbach S. Fidaxomicin versus vancomycin for infection with Clostridium difficile in Europe, Canada, and the USA: a double-blind, non-inferiority, randomised controlled trial. *Lancet Infect Dis* 2012; **12**: 281-289 [PMID: 22321770 DOI: 10.1016/S1473-3099(11)70374-7]

83 **Mullane KM**, Miller MA, Weiss K, Lentnek A, Golan Y, Sears PS, Shue YK, Louie TJ, Gorbach SL. Efficacy of fidaxomicin versus vancomycin as therapy for Clostridium difficile infection in individuals taking concomitant antibiotics for other concurrent infections. *Clin Infect Dis* 2011; **53**: 440-447 [PMID: 21844027 DOI: 10.1093/cid/cir404]

84 **Chaparro-Rojas F**, Mullane KM. Emerging therapies for Clostridium difficile infection - focus on fidaxomicin. *Infect Drug Resist* 2013; **6**: 41-53 [PMID: 23843696 DOI: 10.2147/IDR.S24434]

85 **Bartsch SM**, Umscheid CA, Fishman N, Lee BY. Is fidaxomicin worth the cost? An economic analysis. *Clin Infect Dis* 2013; **57**: 555-561 [PMID: 23704121 DOI: 10.1093/cid/cit346]

86 **Stranges PM**, Hutton DW, Collins CD. Cost-effectiveness analysis evaluating fidaxomicin versus oral vancomycin for the treatment of Clostridium difficile infection in the United States. *Value Health* 2013; **16**: 297-304 [PMID: 23538181 DOI: 10.1016/j.jval.2012.11.004]

87 **Tremaine WJ**. Inflammatory Bowel Disease and Clostridium

difficile-associated diarrhea: a growing problem. *Clin Gastroenterol Hepatol* 2007; **5**: 310-311 [PMID: 17368229]

88 **Issa M,** Weber LR, Skaros S, Beaulieu DB, Emmons J, Knox JF, Lundeen S, Otterson MF, Binion DG. Decreasing rates of colectomy despite high rates of hospitalization in clostridium difficile infected IBD patients: a tertiary referral center experience. *Gastroenterology* 2007; **132**: A663 (abstract)

89 **Clutter DS**, Dubrovskaya Y, Merl MY, Teperman L, Press R, Safdar A. Fidaxomicin versus conventional antimicrobial therapy in 59 recipients of solid organ and hematopoietic stem cell transplantation with clostridium difficile-associated diarrhea. *Antimicrob Agents Chemother* 2013; **57**: 4501-4505 [PMID: 23836168 DOI: 10.1128/AAC.01120-13]

90 **Ben-Horin S**, Margalit M, Bossuyt P, Maul J, Shapira Y, Bojic D, Chermesh I, Al-Rifai A, Schoepfer A, Bosani M, Allez M, Lakatos PL, Bossa F, Eser A, Stefanelli T, Carbonnel F, Katsanos K, Checchin D, Miera IS, Chowers Y, Moran GW. Combination immunomodulator and antibiotic treatment in patients with inflammatory bowel disease and clostridium difficile infection. *Clin Gastroenterol Hepatol* 2009; **7**: 981-987 [PMID: 19523534 DOI: 10.1016/j.cgh.2009.05.031]

91 **Yanai H**, Nguyen GC, Yun L, Lebwohl O, Navaneethan U, Stone CD, Ghazi L, Moayyedi P, Brooks J, Bernstein CN, Ben-Horin S. Practice of gastroenterologists in treating flaring inflammatory bowel disease patients with clostridium difficile: antibiotics alone or combined antibiotics/immunomodulators? *Inflamm Bowel Dis* 2011; **17**: 1540-1546 [PMID: 21674710 DOI: 10.1002/ibd.21514]

92 **MacConnachie AA**, Fox R, Kennedy DR, Seaton RA. Faecal transplant for recurrent Clostridium difficile-associated diarrhoea: a UK case series. *QJM* 2009; **102**: 781-784 [PMID: 19726581 DOI: 10.1093/qjmed/hcp118]

93 **Brandt LJ**, Aroniadis OC, Mellow M, Kanatzar A, Kelly C, Park T, Stollman N, Rohlke F, Surawicz C. Long-term follow-up of colonoscopic fecal microbiota transplant for recurrent Clostridium difficile infection. *Am J Gastroenterol* 2012; **107**: 1079-1087 [PMID: 22450732 DOI: 10.1038/ajg.2012.60]

94 **van Nood E**, Vrieze A, Nieuwdorp M, Fuentes S, Zoetendal EG, de Vos WM, Visser CE, Kuijper EJ, Bartelsman JF, Tijssen JG, Speelman P, Dijkgraaf MG, Keller JJ. Duodenal infusion of donor feces for recurrent Clostridium difficile. *N Engl J Med* 2013; **368**: 407-415 [PMID: 23323867 DOI: 10.1056/NEJMoa1205037]

95 **Anderson JL**, Edney RJ, Whelan K. Systematic review: faecal microbiota transplantation in the management of inflammatory bowel disease. *Aliment Pharmacol Ther* 2012; **36**: 503-516 [PMID: 22827693 DOI: 10.1111/j.1365-2036.2012.05220.x]

96 **Smits LP**, Bouter KE, de Vos WM, Borody TJ, Nieuwdorp M. Therapeutic potential of fecal microbiota transplantation. *Gastroenterology* 2013; **145**: 946-953 [PMID: 24018052 DOI: 10.1053/j.gastro.2013.08.058]

97 **De Leon LM**, Watson JB, Kelly CR. Transient flare of ulcerative colitis after fecal microbiota transplantation for recurrent Clostridium difficile infection. *Clin Gastroenterol Hepatol* 2013; **11**: 1036-1038 [PMID: 23669309 DOI: 10.1016/j.cgh.2013.04.045]

P- Reviewers: Bustamante-Balen M, Li YM, Merlin D, Nielsen OH
S- Editor: Cui XM **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.v19.i43.7586

World J Gastroenterol 2013 November 21; 19(43): 7586-7593
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Extravascular use of drug-eluting beads: A promising approach in compartment-based tumor therapy

Simon Binder, Andrew L Lewis, J-Matthias Löhr, Michael Keese

Simon Binder, Faculty of Medicine Mannheim, University of Heidelberg, 68167 Mannheim, Germany
Andrew L Lewis, Biocompatibles UK Ltd., Department for Research and Development, Farnham GU98QL Surrey, United Kingdom
J-Matthias Löhr, Karolinska Institute, Department of Clinical Science, Intervention and Technology, 17177 Stockholm, Sweden
Michael Keese, Clinic for Vascular and Endovascular Surgery, University Hospital Frankfurt, 60590 Frankfurt am Main, Germany
Author contributions: Binder S and Keese M analyzed the data and wrote the paper; Lewis AL wrote the passage about intrapancreatic therapy; Lewis AL, Löhr JM and Keese M designed and performed the research.
Correspondence to: Michael Keese, PD, MD, Clinic for Vascular and Endovascular Surgery, University Hospital Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt am Main, Germany. michael.keese@kgu.de
Telephone: +49-69-63015349 Fax: +49-69-63015336
Received: August 11, 2013 Revised: September 5, 2013
Accepted: September 16, 2013
Published online: November 21, 2013

Abstract

Intraperitoneal carcinomatosis (PC) may occur with several tumor entities. The prognosis of patients suffering from PC is usually poor. Present treatment depends on the cancer entity and includes systemic chemotherapy, radiation therapy, hormonal therapy and surgical resection. Only few patients may also benefit from hyperthermic intraperitoneal chemotherapy with a complete tumor remission. These therapies are often accompanied by severe systemic side-effects. One approach to reduce side effects is to target chemotherapeutic agents to the tumor with carrier devices. Promising experimental results have been achieved using drug-eluting beads (DEBs). A series of *in vitro* and *in vitro* experiments has been conducted to determine the suitability of their extravascular use. These encapsulation devices were able to harbor CYP2B1 producing cells and to shield them from the hosts immune system when injected intratumorally. In this way ifosfamide - which is transformed into its active metabolites by CYP2B1 - could be successfully targeted into pancreatic tumor growths. Furthermore DEBs can be used to target chemotherapeutics into the abdominal cavity for treatment of PC. If CYP2B1 producing cells are proven to be save for usage in man and if local toxic effects of chemotherapeutics can be controlled, DEBs will become promising tools in compartment-based anticancer treatment.

Key words: Compartment based therapy; Intraperitoneal; Drug-eluting beads; Carcinomatosis; Hyperthermic intraperitoneal chemotherapy; Glioblastoma; Pancreatic cancer; CYP2B1; Ifosfamide

Core tip: Intraperitoneal carcinomatosis occurs with several tumor entities and prognosis is usually poor. Besides standard therapy, only few patients may benefit from hyperthermic intraperitoneal chemotherapy. The treatment may cause severe systemic side-effects. One different approach to target chemotherapeutic agents to the tumor employs carrier devices. Contemplable carriers are drug-eluting beads (DEBs). DEBs can be used to transfer drugs or pro-drug converting enzymes directly to the tumor. Furthermore, DEBs can successfully target chemotherapeutics into the abdominal cavity for *ip* treatment. When local toxic effects are controlled, DEBs are effective tools in compartment-based therapy.

Binder S, Lewis AL, Löhr JM, Keese M. Extravascular use of drug-eluting beads: A promising approach in compartment-based tumor therapy. *World J Gastroenterol* 2013; 19(43): 7586-7593 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7586.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7586

CLINICAL BACKGROUND

Peritoneal carcinomatosis (PC) is a disseminated tumor stage, which is observed in patients with ovarian, pancreatic, gastric and colorectal cancer. With median survival rates of 3.1 mo for gastric cancer and 5.2 mo for colorectal cancer, respectively[1], the prognosis is usually poor[2].

Survival is prolonged by new agents used in palliative chemotherapy. With the availability of oxaliplatin, irinotecan, bevacizumab, and cetuximab the 5-year survival has significantly increased over the last decade[3-7].

Although some patients seem to benefit from these drugs, the physical and psychological strain for patients suffering from PC remains high. In addition to the commonly known side effects of chemotherapy[8], patients show a variety of symptoms originating from PC itself, ranging from abdominal pain, nausea and obstipation up to bowel obstruction and obstructive uropathy[9].

EVALUATION OF PRESENT TREATMENT

The treatment of peritoneal carcinomatosis requires an interdisciplinary and multimodal approach. Modern therapy combines cytoreductive surgery (CRS), radiation therapy and systemic chemotherapy, depending on the origin of the tumor[10-12]. Unfortunately survival rates remain low[1,13] at the cost of frequently observed dose limiting side-effects[8].

Since tumor spread into the abdominal cavity may also be considered as an early step of dissemination - comparable to liver metastasis in colorectal carcinoma - and not as a state of generalized systemic disease[14-16], one approach may be to resect all detectable tumor nodules and target drugs directly to the peritoneal cavity[17].

The surgical procedure removes all macroscopic tumor manifestations by combination of different peritonectomy procedures, including greater omentectomy, splenectomy, left upper quadrant peritonectomy, right upper quadrant peritonectomy, lesser omentectomy, cholecystectomy with stripping of the omental bursa, pelvic peritonectomy with sleeve resection of the sigmoid colon, and antrectomy[18-21], as well as parietal peritonectomy[22,23]. After the resection, the dissolved chemotherapeutic agent and carrier solution are heated up to 42 ℃ and pumped through the abdominal cavity for 40-90 min[24]. Since the abdomen remains opened, it is possible for the surgeon to support the circulation in the abdominal cavity manually[25]. This procedure is followed by thorough lavage, anastomosis of resected bowel segments and closure of the abdominal wall[24].

A survival benefit using CRS and hyperthermic intraperitoneal chemotherapy (HIPEC) has been shown[26]. Verwaal *et al*[27] reported a 3-year survival of 38% in their patients. A more recent follow up of the same cohort shows similar survival rates[28]. Median progression-free survival was 7.7 mo in the control arm and 12.6 mo in the HIPEC arm. A 5-year survival of 45% was found in patients, in which R1 resection could be achieved. This indicates that CRS combined with HIPEC is superior to systemic chemotherapy alone. Nevertheless the findings of Franko *et al*[29] suggest, that CRS combined with HIPEC as well as systemic chemotherapy alone have their roles in the multidisciplinary approach treating peritoneally disseminated cancer.

In selected patients even a long term survival may be possible, with CRS and HIPEC being a curative approach in disseminated colorectal carcinoma[28,30].

The HIPEC procedure itself is demanding for most of the patients. Even though the median survival rates increased, the 30-d mortality rate of 4.8% and a morbidity rate reaching up to 55% are high[31]. The surgery itself and severe systemic side-effects may lead to deterioration of health or death[32,33]. Given that, the inclusion criteria to receive CRS and HIPEC remain strict. The peritoneal surface has to be the only site of disease dissemination[27] and the preoperative assessment[34] should suggest a high likelihood of achieving complete cytoreduction (CC-0)[35]. Therefore only patients with medium-sized intraperitoneal tumor nodules and a limited distribution within the abdomen are selected[36]. Patients have to be physically fit to endure this extensive procedure. Considering that peritoneal carcinomatosis only becomes symptomatic in advanced stages, where CC-0 or CC-1 can rarely be achieved, only few highly selected patients have access to this approach[37]. Excluded patients are left with systemic chemotherapy.

Alternative techniques have been investigated to target chemotherapeutic agents to the body cavities without the strain of surgery. These are promising approaches to circumvent both the systemic side effects and the hazard of an extensive surgical procedure.

DRUG-ELUTING BEADS

Bead characteristics

Promising carriers for contemplable agents such as doxorubicin, irinotecan or mitoxantrone are drug-eluting beads (DEB).

By far the most commonly used product in clinic is DC Bead™, which are microspheres comprised of a sulphonate-modified polyvinyl-alcohol hydrogel. They are available in sizes from 70-700 μm[38] and can be loaded with doxorubicin (DOX), irinotecan (IRI) or mitoxantrone (MTX)[39]. When drug-loaded, the product provides an accurate dosage of drug per unit volume of beads *in vitro*[38], which they release *via* ion exchange constantly over weeks[40,41]. *In vitro,* the beads are robust and maintain their size and shape after drug loading[42]. This is a prerequisite for DEBs, since damage of the beads may cause rapid liberation and significant systemic distribution of the encapsulated drug or adverse effects by the debris itself.

The surface of the DEBs itself is inert and did not cause any immune reaction in control groups treated with unloaded beads[39,43]. Furthermore the biomechanical engineered material is able to shield its content from the immune system[44,45].

Present field of application

DEBs are used in clinical practice for trans-arterial chemoembolisation (TACE) of hypervascularized tumors[46], such as hepatocellular carcinoma (HCC) and liver metastasis. By administering them selectively into the tumor-feeding vessels, the route for essential nutrients is obstructed and high levels of antineoplastic drugs can be reached within the tumor[47].

As the procedure itself can be carried out under local anesthesia, morbidity and complication rates are low[48], TACE has become the standard palliative approach in patients with unresectable HCC[49-51]. The objective response rates range from 70%-75%[52,53] at a low rate of complications[53]. This suggests a good risk-benefit ratio.

For both associated side effects[54] and progression free survival[55] as well as overall disease control[54,55], doxorubicin-loaded DC beads (DOXDEB™) produced the most promising results.

DEBS IN EXTRAVASCULAR USE

Since DEBs are able to liberate agents continuously *in vitro*[56] they can also serve as drug carriers for extravascular application if the beads are directly instilled into the compartments.

Intracerebal therapy

The median survival of rats with experimental glioblastoma multiforme (GBM) could be successfully prolonged using doxorubicin polymers[57]. This demonstrated a superior effect of chemotherapeutic carriers as compared to *iv* administration of the free drug. The most efficient drug - namely doxorubicin - caused the most severe side effects. Intracerebral hemorrhage and edema as well as hemiparesis were observed[57]. A significantly longer median survival could be achieved in patients with GBM using carmustine warfers[58], but they did not affect the recurrence-free survival times[59]. These findings justified the idea of compartment-based therapy, but also called for new delivery systems and alternative antineoplastic drugs in return.

Baltes *et al*[60] showed that the intracerebral administration of DEBs is safe for use depending on the loaded drug. Both doxorubicin- and irinotecan-loaded DEBs significantly improved survival time in a rat BT4Ca GBM model. Doxorubicin again caused severe side effects[57] whereas irinotecan seemed to selectively affect only the cancer cells and not healthy brain tissue[61,62]. These findings could be confirmed in follow-up experiments where alginate was used as a viscosity modifier to secure the administration of the beads into the tissue[63].

Intrapancreatic therapy

The efficacy of irinotecan- and topotecan-loaded DEBs have been evaluated by use of a modified MTS assay and in a PSN-1 mouse xenograft model of pancreatic cancer by direct injection at the tumor site. Topotecan was shown to be more potent than irinotecan in the *in vitro* cell assay, had reasonable efficacy and tolerability at 0.2-0.4 mg doses but was lethal at doses of 0.83-1.2 mg. Irinotecan however, was well tolerated even with repeated injections of doses from 3.3-6.6 mg and displayed good efficacy[64]. A similar study evaluated combinations of doxorubicin, irinotecan, topotecan and rapamycin DEBs and demonstrated synergistic activity for certain drug combinations, in particular doxorubicin and rapamycin[65].

Feasibility for the clinical application of the direct intratumoral delivery of a compartment-based therapy was first demonstrated by delivery of a reservoir of a thermosensitive gel containing paclitaxel (Oncogel®) into the pancreas by use of ultrasound-guided endoscopic needle injection[66,67]. This approach has been subsequently adapted for the administration of irinotecan-loaded DEBs suspended in alginate into the tail of the pancreas of a healthy pig. The therapy was well tolerated up to doses of 300 mg of irinotecan, with only localized pancreatic tissue reactions on histopathologic review[68].

Intraperitoneal therapy of peritoneal carcinomatosis

An elegant approach to target drugs to a tumor is to administer them as pro-drugs and activate them intratumorally. The active metabolites are formed by enzymes which are selectively injected into the tumor.

Routinely, ifosfamide has been used *via iv* application in pancreatic cancer treatment[69,70]. After administration cytochrome P450 2B1 (CYP2B1) produced by hepatocytes, transforms ifosfamide into 4-OH-ifosfamide, which results in the active compounds phosphoramide mustard and acrolein[71]. *In vitro* preparation and direct administration of the active compounds are limited due to their short half life (45 min)[72].

Löhr *et al*[73] used encapsulated feline kidney cells, engineered to produce CYP2B1[74], to target activated ifosfamide into pancreatic carcinoma[73]. Therefore, cells were encapsulated in cellulose sulphate[75] for immobilization and to protect them from the immune system when injected into the tumor. To model a pancreatic cell-like carcinoma PaCa-44 human pancreatic tumor cells were injected subcutaneously into nude mice. All mice received ifosfamide *iv*, one group received intra-tumorous injection of encapsulated CYP2B1 producing cells and one group received nonencapsulated cells. Tumor growth was impaired in all mice receiving ifosfamide. However, the most significant tumor reduction was detectable in the group that had received encapsulated cells. Complete macro- and microscopic tumor remission could be achieved in 20% of the animals. Although the same dosages of ifosfamide were used in both groups, the apoptotic rate of tumor cells was three times higher in the group receiving encapsulated cells. Furthermore these animals appeared healthier than the ones receiving nonencapsulated cells. Müller *et al*[43] were able to reproduce these results using a CYP2B1 producing cell line of human origin. This cell line did not produce potentially harmful retroviruses[76] and is immune resistant[77].

The approach worked for other tumor entities as

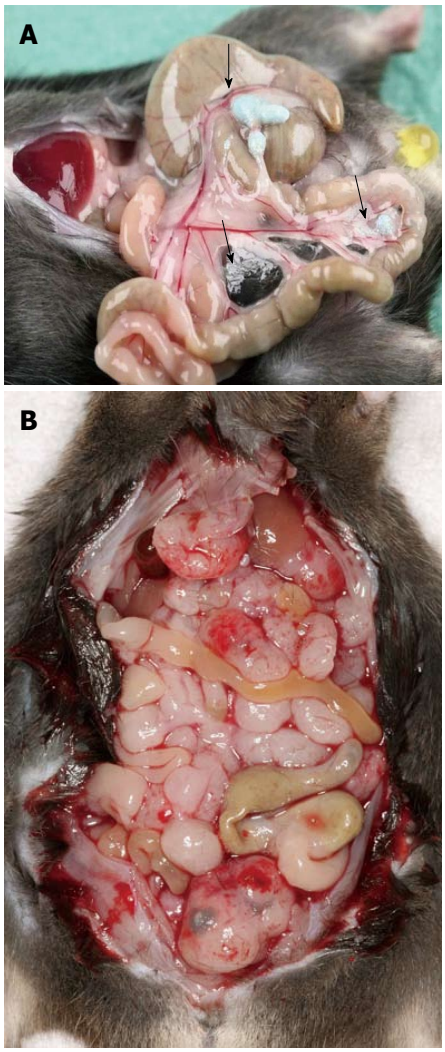


Figure 1 Peritoneal metastasis. A: Beads accumulate in the mesentery of the small bowel (arrors). Animals show a complete tumor remission; B: Control animal with disseminated peritoneal carcinomatosis induced by EGFP-C-26 cells.

well. Samel *et al*[78] showed, that similar results could be achieved in Balb/c mice carrying peritoneal tumor nodules, induced by syngenic C-26 cells injected into the abdominal cavity. This cell line is highly malignant and rapidly forms tumor nodules on the peritoneum. Again, in some animals a complete response was achieved. One major drawback of this approach is the use of genetically engineered cells. These cells may maintain a malignant potential. It remains to be shown if they can be safely applied to patients.

Therefore, an easier approach directly employs encapsulated chemo agents. *In vitro* tests with wild-type C-26 murine colon-carcinoma cells showed potent tumor toxicity for free DOX, IRI and MTX and the encapsulated drugs when combinations of the chemotherapeutic agents and DEBs were tested[39]. For free IRI and MTX the inhibition of cell growth was superior to their encapsulated forms. The proportion of apoptotic cells was significantly higher for free DOX as well as for DOXDEB ™ when compared to the other two agents. Both DOX and MTX showed a dose-depending induction of apoptosis, whereas IRI did not show any significant effect.

In vivo tests followed after determining appropriate concentration levels[39]. For better detection of micrometastases and for tumor load quantification, C-26 cells had been transfected with the marker protein enhanced green florescent protein as described[78,79]. All animals developed disseminated PC (Figure 1A). Thereafter, animals were treated with free and encapsulated DOX and MTX. Best tumor reduction was obtained when splitting the DEB application into three sessions. Complete tumor remission could be obtained (Figure 1B). Weight loss and mortality of the subjects was significantly higher in the groups which were treated with the corresponding free drugs, suggesting a lower toxicity in the DEB groups.

The results obtained in this model of colorectal tumor, could be reproduced for pancreatic carcinoma dissemination. Yagublu *et al*[80] used a model of peritoneally metastasized panc02 pancreatic carcinoma cells in C57 black6 mice. Treatment was performed with free and encapsulated DOX, IRI and MTX. The free drug was more potent in decreasing tumor cell growth and inducing apoptosis than the encapsulated drugs *in vitro*. Again, *in vivo* free drug administration caused more weight loss and significantly higher lethality than the encapsulated drug, while no relevant differences in antitumoral activity could be observed.

To test the safety of the intraperitoneal injection and therapy using the DOXDEB™ a large animal trail was carried out[81]. Black-headed meat-sheep received an application of DOXDEB™ into the abdominal cavity. Up to 50% of the maximal cumulative dose suggested for male humans were used in one single intraperitoneal injection[82]. DEBs were injected using a verres needle. Upon autopsy, no DEBs were distributed *via* blood or lymphatic vessels. Beads remained on the peritoneum, immobilized by a fibrin layer (Figure 2A and B). No evidence for organ-related damage or systemic toxicity was observed. This is remarkable, as cardio toxicity[83-87] and myelosuppresion[88-91] are frequently described with the systemic use of doxorubicin, along with less severe side effects such as stomatitis, alopecia, nausea and vomiting[90]. The systemic distribution of DOX followed a three-compartment-model omitting a rapid and high peak, in comparison to *iv* administration. Serum levels reached a steady-state 360 min after application with a half-life of 615 h. Some sheep did not reach the end point and developed a chemical peritonitis[82,92,93] (Figure 3). By circumventing the systemic administration and its accompanying side effects, local toxicity was the only limiting factor. This underlines the importance of drug choice when it comes to DEB therapy within the intraperitoneal compartment.

CONCLUSION

There is convincing evidence that drug-eluting beads can be employed in an extravascular environment for a compartment-based therapy. In several tumor models, the carrier devices showed convincing tumor control and

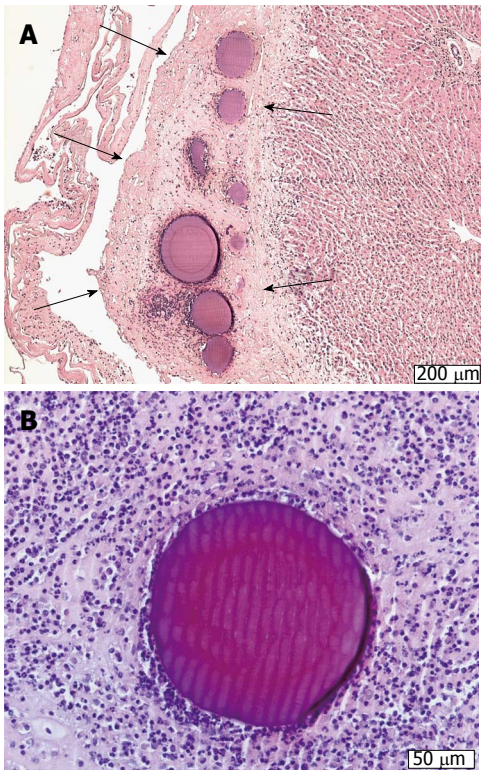


Figure 2 Doxorubicin-eluting beads. A: HE-stained, magnification × 50: A layer of fibrin (between arrows) immobilizing the doxorubicin-loaded DC beads (DOXDEB™) on the livers surface; B: HE-stained, magnification × 100: DOXDEB™ in layer of fibrin and surrounded by lymphocytes immobilizing it on mesenteric connective tissue.

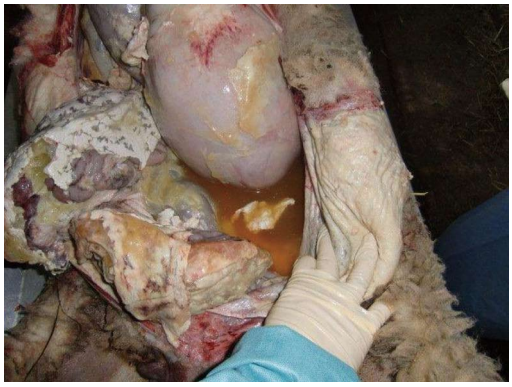


Figure 3 Chemical peritonitis. Autopsy of an animal 28 d after installation of *ip* doxorubicin-loaded drug-eluting beads: Situs with greater omentum and intestinal loops with fibrinous adhesions, amber-colored ascitic fluid.

side-effects were less likely to occur. Also, encapsulation devices can be used to transform pro-drugs into their active metabolites within or in vicinity of the tumor. Here, drug-eluting beads successfully immobilized transformating-enzyme producing cells and protected them from the host immune system. However, the application of genetically engineered cell lines remains a major safety concern.

Intraperitoneal application of DEBs is a small procedure which can be safely performed under local anesthesia. Within the abdominal cavity DEBs show predictable liberation characteristics, remain inert and do not distribute *via* blood or lymphatic vessels.

Compartment-based therapy could be considered as a favorable treatment option for palliative patients with a deteriorated general condition, who are not eligible for HIPEC. Local toxicity is a limiting factor. Other drugs - for example irinotecan - have to be tested in a large animal model to further investigate local reactions.

If the adverse effects of the loaded substances are controlled, the extravascular use of drug-eluting beads is a promising future approach in compartment-based tumor therapy.

REFERENCES

1 **Sadeghi B**, Arvieux C, Glehen O, Beaujard AC, Rivoire M, Baulieux J, Fontaumard E, Brachet A, Caillot JL, Faure JL, Porcheron J, Peix JL, François Y, Vignal J, Gilly FN. Peritoneal carcinomatosis from non-gynecologic malignancies: results of the EVOCAPE 1 multicentric prospective study. *Cancer* 2000; **88**: 358-363 [PMID: 10640968]

2 **Tischoff I**, Tannapfel A. Pathologic and anatomic evidence of peritoneal metastases. *Chirurg* 2007; **78**: 1085-1086, 1088-1090 [PMID: 18030433 DOI: 10.1007/s00104-007-1427-0]

3 **Dy GK**, Krook JE, Green EM, Sargent DJ, Delaunoit T, Morton RF, Fuchs CS, Ramanathan RK, Williamson SK, Findlay BP, Pockaj BA, Sticca RP, Alberts SR, Pitot HC, Goldberg RM. Impact of complete response to chemotherapy on overall survival in advanced colorectal cancer: results from Intergroup N9741. *J Clin Oncol* 2007; **25**: 3469-3474 [PMID: 17687151 DOI: 10.1200/JCO.2007.10.7128]

4 **Grothey A**, Sargent D. Overall survival of patients with advanced colorectal cancer correlates with availability of fluorouracil, irinotecan, and oxaliplatin regardless of whether doublet or single-agent therapy is used first line. *J Clin Oncol* 2005; **23**: 9441-9442 [PMID: 16361649 DOI: 10.1200/JCO.2005.04.4792]

5 **Grothey A**, Sugrue MM, Purdie DM, Dong W, Sargent D, Hedrick E, Kozloff M. Bevacizumab beyond first progression is associated with prolonged overall survival in metastatic colorectal cancer: results from a large observational cohort study (BRiTE). *J Clin Oncol* 2008; **26**: 5326-5334 [PMID: 18854571 DOI: 10.1200/JCO.2008.16.3212]

6 **Van Cutsem E**, Bajetta E, Valle J, Köhne CH, Hecht JR, Moore M, Germond C, Berg W, Chen BL, Jalava T, Lebwohl D, Meinhardt G, Laurent D, Lin E. Randomized, placebo-controlled, phase III study of oxaliplatin, fluorouracil, and leucovorin with or without PTK787/ZK 222584 in patients with previously treated metastatic colorectal adenocarcinoma. *J Clin Oncol* 2011; **29**: 2004-2010 [PMID: 21464401 DOI: 10.1200/JCO.2010.29.5436]

7 **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]

8 **Griffin AM**, Butow PN, Coates AS, Childs AM, Ellis PM, Dunn SM, Tattersall MH. On the receiving end. V: Patient perceptions of the side effects of cancer chemotherapy in 1993. *Ann Oncol* 1996; **7**: 189-195 [PMID: 8777177]

9 **Carraro PG**, Segala M, Cesana BM, Tiberio G. Obstructing colonic cancer: failure and survival patterns over a ten-year follow-up after one-stage curative surgery. *Dis Colon Rectum* 2001; **44**: 243-250 [PMID: 11227942]

10 **Schmiegel W**, Reinacher-Schick A, Arnold D, Graeven U,

Heinemann V, Porschen R, Riemann J, Rödel C, Sauer R, Wieser M, Schmitt W, Schmoll HJ, Seufferlein T, Kopp I, Pox C. Update S3-guideline "colorectal cancer" 2008. *Z Gastroenterol* 2008; **46**: 799-840 [PMID: 18759205 DOI: 10.1055/s-2008-1027726]

11 **Schmalfeldt B**, Pfisterer, J. Interdisziplinäre S2k-Leitlinie für die Diagnostik und Therapie maligner Ovarialtumoren. Kommission Ovar der Arbeitsgemeinschaft Gynäkologische Onkologie eV. München, Wien, New York: W. Zuckschwerdt Verlag GmbH, 2007

12 **Weller M**. Interdisziplinäre S 2 – Leitlinie für die Diagnostik und Therapie der Gliome des Erwachsenenalters. In: Neurology GSo, ed. 030/099. Germany: AWMF online, 2011

13 **Affronti ML**, Heery CR, Herndon JE, Rich JN, Reardon DA, Desjardins A, Vredenburgh JJ, Friedman AH, Bigner DD, Friedman HS. Overall survival of newly diagnosed glioblastoma patients receiving carmustine wafers followed by radiation and concurrent temozolomide plus rotational multiagent chemotherapy. *Cancer* 2009; **115**: 3501-3511 [PMID: 19514083 DOI: 10.1002/cncr.24398]

14 **Sugarbaker PH**. Intraperitoneal chemotherapy and cytoreductive surgery for the prevention and treatment of peritoneal carcinomatosis and sarcomatosis. *Semin Surg Oncol* 1998; **14**: 254-261 [PMID: 9548609]

15 **Sugarbaker PH**. Management of peritoneal-surface malignancy: the surgeon's role. *Langenbecks Arch Surg* 1999; **384**: 576-587 [PMID: 10654274]

16 **Glehen O**, Osinsky D, Beaujard AC, Gilly FN. Natural history of peritoneal carcinomatosis from nongynecologic malignancies. *Surg Oncol Clin N Am* 2003; **12**: 729-739, xiii [PMID: 14567027]

17 **Fernández-Trigo V**, Stuart OA, Stephens AD, Hoover LD, Sugarbaker PH. Surgically directed chemotherapy: heated intraperitoneal lavage with mitomycin C. *Cancer Treat Res* 1996; **81**: 51-61 [PMID: 8834575]

18 **Sugarbaker PH**. Peritonectomy procedures. *Ann Surg* 1995; **221**: 29-42 [PMID: 7826158]

19 **Sugarbaker PH**. Peritonectomy procedures. *Cancer Treat Res* 1996; **82**: 235-253 [PMID: 8849954]

20 **Sugarbaker PH**. Peritonectomy procedures. *Surg Oncol Clin N Am* 2003; **12**: 703-727, xiii [PMID: 14567026]

21 **Sugarbaker PH**. Peritonectomy procedures. *Cancer Treat Res* 2007; **134**: 247-264 [PMID: 17633058]

22 **Vazquez Vde L**, Sugarbaker PH. Total anterior parietal peritonectomy. *J Surg Oncol* 2003; **83**: 261-263 [PMID: 12884240 DOI: 10.1002/jso.10277]

23 **Sugarbaker PH**. Parietal peritonectomy. *Ann Surg Oncol* 2012; **19**: 1250 [PMID: 22322950 DOI: 10.1245/s10434-012-2229-2]

24 **Kerscher A**. Die HIPEC - hypertherme intraperitoneale Chemoperfusion. Würzburg: Peritonealkarzinosezentrum Würzburg, Chirurgische Klinik der Universitätsklinik Würzburg, 2010

25 **Stephens AD**, Alderman R, Chang D, Edwards GD, Esquivel J, Sebbag G, Steves MA, Sugarbaker PH. Morbidity and mortality analysis of 200 treatments with cytoreductive surgery and hyperthermic intraoperative intraperitoneal chemotherapy using the coliseum technique. *Ann Surg Oncol* 1999; **6**: 790-796 [PMID: 10622509]

26 **Sugarbaker PH**, Jablonski KA. Prognostic features of 51 colorectal and 130 appendiceal cancer patients with peritoneal carcinomatosis treated by cytoreductive surgery and intraperitoneal chemotherapy. *Ann Surg* 1995; **221**: 124-132 [PMID: 7857141]

27 **Verwaal VJ**, van Ruth S, de Bree E, van Sloothen GW, van Tinteren H, Boot H, Zoetmulder FA. Randomized trial of cytoreduction and hyperthermic intraperitoneal chemotherapy versus systemic chemotherapy and palliative surgery in patients with peritoneal carcinomatosis of colorectal cancer. *J Clin Oncol* 2003; **21**: 3737-3743 [PMID: 14551293 DOI: 10.1200/JCO.2003.04.187]

28 **Verwaal VJ**, Bruin S, Boot H, van Slooten G, van Tinteren H. 8-year follow-up of randomized trial: cytoreduction and hyperthermic intraperitoneal chemotherapy versus systemic chemotherapy in patients with peritoneal carcinomatosis of colorectal cancer. *Ann Surg Oncol* 2008; **15**: 2426-2432 [PMID: 18521686 DOI: 10.1245/s10434-008-9966-2]

29 **Franko J**, Ibrahim Z, Gusani NJ, Holtzman MP, Bartlett DL, Zeh HJ. Cytoreductive surgery and hyperthermic intraperitoneal chemoperfusion versus systemic chemotherapy alone for colorectal peritoneal carcinomatosis. *Cancer* 2010; **116**: 3756-3762 [PMID: 20564081 DOI: 10.1002/cncr.25116]

30 **Sugarbaker PH**. A curative approach to peritoneal carcinomatosis from colorectal cancer. *Semin Oncol* 2005; **32**: S68-S73 [PMID: 16399436 DOI: 10.1053/j.seminoncol.2005.06.009]

31 **Gill RS**, Al-Adra DP, Nagendran J, Campbell S, Shi X, Haase E, Schiller D. Treatment of gastric cancer with peritoneal carcinomatosis by cytoreductive surgery and HIPEC: a systematic review of survival, mortality, and morbidity. *J Surg Oncol* 2011; **104**: 692-698 [PMID: 21713780 DOI: 10.1002/jso.22017]

32 **Glockzin G**, Ghali N, Lang SA, Agha A, Schlitt HJ, Piso P. Peritoneal carcinomatosis. Surgical treatment, including hyperthermal intraperitoneal chemotherapy. *Chirurg* 2007; **78**: 1100, 1102-1106, 1108-1110 [PMID: 17992490 DOI: 10.1007/s00104-007-1419-0]

33 **Glockzin G**, Schlitt HJ, Piso P. Peritoneal carcinomatosis: patients selection, perioperative complications and quality of life related to cytoreductive surgery and hyperthermic intraperitoneal chemotherapy. *World J Surg Oncol* 2009; **7**: 5 [PMID: 19133112 DOI: 10.1186/1477-7819-7-5]

34 **Cavaliere F**, Perri P, Rossi CR, Pilati PL, De Simone M, Vaira M, Deraco M, Di Filippo F. Indications for integrated surgical treatment of peritoneal carcinomatosis of colorectal origin: experience of the Italian Society of Locoregional Integrated Therapy in Oncology. *Tumori* 2003; **89**: 21-23 [PMID: 12903536]

35 **Glehen O**, Cotte E, Schreiber V, Sayag-Beaujard AC, Vignal J, Gilly FN. Intraperitoneal chemohyperthermia and attempted cytoreductive surgery in patients with peritoneal carcinomatosis of colorectal origin. *Br J Surg* 2004; **91**: 747-754 [PMID: 15164446 DOI: 10.1002/bjs.4473]

36 **Salti GI**, Ailabouni L, Undevia S. Cytoreductive surgery and hyperthermic intraperitoneal chemotherapy for the treatment of peritoneal sarcomatosis. *Ann Surg Oncol* 2012; **19**: 1410-1415 [PMID: 22302269 DOI: 10.1245/s10434-012-2240-7]

37 **Esquivel J**, Sticca R, Sugarbaker P, Levine E, Yan TD, Alexander R, Baratti D, Bartlett D, Barone R, Barrios P, Bieligk S, Bretcha-Boix P, Chang CK, Chu F, Chu Q, Daniel S, de Bree E, Deraco M, Dominguez-Parra L, Elias D, Flynn R, Foster J, Garofalo A, Gilly FN, Glehen O, Gomez-Portilla A, Gonzalez-Bayon L, Gonzalez-Moreno S, Goodman M, Gushchin V, Hanna N, Hartmann J, Harrison L, Hoefer R, Kane J, Kecmanovic D, Kelley S, Kuhn J, Lamont J, Lange J, Li B, Loggie B, Mahteme H, Mann G, Martin R, Misih RA, Moran B, Morris D, Onate-Ocana L, Petrelli N, Philippe G, Pingpank J, Pitroff A, Piso P, Quinones M, Riley L, Rutstein L, Saha S, Alrawi S, Sardi A, Schneebaum S, Shen P, Shibata D, Spellman J, Stojadinovic A, Stewart J, Torres-Melero J, Tuttle T, Verwaal V, Villar J, Wilkinson N, Younan R, Zeh H, Zoetmulder F, Sebbag G. Cytoreductive surgery and hyperthermic intraperitoneal chemotherapy in the management of peritoneal surface malignancies of colonic origin: a consensus statement. Society of Surgical Oncology. *Ann Surg Oncol* 2007; **14**: 128-133 [PMID: 17072675 DOI: 10.1245/s10434-006-9185-7]

38 **Lewis AL**, Gonzalez MV, Lloyd AW, Hall B, Tang Y, Willis SL, Leppard SW, Wolfenden LC, Palmer RR, Stratford PW. DC bead: in vitro characterization of a drug-delivery device

for transarterial chemoembolization. *J Vasc Interv Radiol* 2006; **17**: 335-342 [PMID: 16517780]

39 **Keese M**, Gasimova L, Schwenke K, Yagublu V, Shang E, Faissner R, Lewis A, Samel S, Löhr M. Doxorubicin and mitoxantrone drug eluting beads for the treatment of experimental peritoneal carcinomatosis in colorectal cancer. *Int J Cancer* 2009; **124**: 2701-2708 [PMID: 19165866 DOI: 10.1002/ijc.24211]

40 **Taylor RR**, Tang Y, Gonzalez MV, Stratford PW, Lewis AL. Irinotecan drug eluting beads for use in chemoembolization: in vitro and in vivo evaluation of drug release properties. *Eur J Pharm Sci* 2007; **30**: 7-14 [PMID: 17030118 DOI: 10.1016/j.ejps.2006.09.002]

41 **Gonzalez MV**, Tang Y, Phillips GJ, Lloyd AW, Hall B, Stratford PW, Lewis AL. Doxorubicin eluting beads-2: methods for evaluating drug elution and in-vitro: in-vivo correlation. *J Mater Sci Mater Med* 2008; **19**: 767-775 [PMID: 17653626 DOI: 10.1007/s10856-006-0040-y]

42 **Jordan O**, Denys A, De Baere T, Boulens N, Doelker E. Comparative study of chemoembolization loadable beads: in vitro drug release and physical properties of DC bead and hepasphere loaded with doxorubicin and irinotecan. *J Vasc Interv Radiol* 2010; **21**: 1084-1090 [PMID: 20610183 DOI: 10.1016/j.jvir.2010.02.042]

43 **Müller P**, Jesnowski R, Karle P, Renz R, Saller R, Stein H, Püschel K, von Rombs K, Nizze H, Liebe S, Wagner T, Günzburg WH, Salmons B, Löhr M. Injection of encapsulated cells producing an ifosfamide-activating cytochrome P450 for targeted chemotherapy to pancreatic tumors. *Ann N Y Acad Sci* 1999; **880**: 337-351 [PMID: 10415878]

44 **Löhr M**, Hoffmeyer A, Kröger J, Freund M, Hain J, Holle A, Karle P, Knöfel WT, Liebe S, Müller P, Nizze H, Renner M, Saller RM, Wagner T, Hauenstein K, Günzburg WH, Salmons B. Microencapsulated cell-mediated treatment of inoperable pancreatic carcinoma. *Lancet* 2001; **357**: 1591-1592 [PMID: 11377651]

45 **Okada N**, Miyamoto H, Yoshioka T, Sakamoto K, Katsume A, Saito H, Nakagawa S, Ohsugi Y, Mayumi T. Immunological studies of SK2 hybridoma cells microencapsulated with alginate-poly(L)lysine-alginate (APA) membrane following allogeneic transplantation. *Biochem Biophys Res Commun* 1997; **230**: 524-527 [PMID: 9015354 DOI: 10.1006/bbrc.1996.5996]

46 **Aliberti C**, Benea G, Tilli M, Fiorentini G. Chemoembolization (TACE) of unresectable intrahepatic cholangiocarcinoma with slow-release doxorubicin-eluting beads: preliminary results. *Cardiovasc Intervent Radiol* 2008; **31**: 883-888 [PMID: 18478290 DOI: 10.1007/s00270-008-9336-2]

47 **Lencioni R**, de Baere T, Burrel M, Caridi JG, Lammer J, Malagari K, Martin RC, O'Grady E, Real MI, Vogl TJ, Watkinson A, Geschwind JF. Transcatheter treatment of hepatocellular carcinoma with Doxorubicin-loaded DC Bead (DEBDOX): technical recommendations. *Cardiovasc Intervent Radiol* 2012; **35**: 980-985 [PMID: 22009576 DOI: 10.1007/s00270-011-0287-7]

48 **Skowasch M**, Schneider J, Otto G, Weinmann A, Woerns MA, Dueber C, Pitton MB. Midterm follow-up after DC-BEAD™-TACE of hepatocellular carcinoma (HCC). *Eur J Radiol* 2012; **81**: 3857-3861 [PMID: 22840383 DOI: 10.1016/j.ejrad.2012.07.002]

49 **Lo CM**, Ngan H, Tso WK, Liu CL, Lam CM, Poon RT, Fan ST, Wong J. Randomized controlled trial of transarterial lipiodol chemoembolization for unresectable hepatocellular carcinoma. *Hepatology* 2002; **35**: 1164-1171 [PMID: 11981766 DOI: 10.1053/jhep.2002.33156]

50 **Llovet JM**, Real MI, Montaña X, Planas R, Coll S, Aponte J, Ayuso C, Sala M, Muchart J, Solà R, Rodés J, Bruix J. Arterial embolisation or chemoembolisation versus symptomatic treatment in patients with unresectable hepatocellular carcinoma: a randomised controlled trial. *Lancet* 2002; **359**: 1734-1739 [PMID: 12049862 DOI: 10.1016/S0140-6736(02)08649-X]

51 **Llovet JM**, Bruix J. Systematic review of randomized trials for unresectable hepatocellular carcinoma: Chemoembolization improves survival. *Hepatology* 2003; **37**: 429-442 [PMID: 12540794 DOI: 10.1053/jhep.2003.50047]

52 **Varela M**, Real MI, Burrel M, Forner A, Sala M, Brunet M, Ayuso C, Castells L, Montañá X, Llovet JM, Bruix J. Chemoembolization of hepatocellular carcinoma with drug eluting beads: efficacy and doxorubicin pharmacokinetics. *J Hepatol* 2007; **46**: 474-481 [PMID: 17239480 DOI: 10.1016/j.jhep.2006.10.020]

53 **Malagari K**, Chatzimichael K, Alexopoulou E, Kelekis A, Hall B, Dourakis S, Delis S, Gouliamos A, Kelekis D. Transarterial chemoembolization of unresectable hepatocellular carcinoma with drug eluting beads: results of an open-label study of 62 patients. *Cardiovasc Intervent Radiol* 2008; **31**: 269-280 [PMID: 17999110 DOI: 10.1007/s00270-007-9226-z]

54 **Martin R**, Geller D, Espat J, Kooby D, Sellars M, Goldstein R, Imagawa D, Scoggins C. Safety and efficacy of trans arterial chemoembolization with drug-eluting beads in hepatocellular cancer: a systematic review. *Hepatogastroenterology* 2012; **59**: 255-260 [PMID: 22251546 DOI: 10.5754/hge10240]

55 **Song MJ**, Park CH, Kim JD, Kim HY, Bae SH, Choi JY, Yoon SK, Chun HJ, Choi BG, Lee HG. Drug-eluting bead loaded with doxorubicin versus conventional Lipiodol-based transarterial chemoembolization in the treatment of hepatocellular carcinoma: a case-control study of Asian patients. *Eur J Gastroenterol Hepatol* 2011; **23**: 521-527 [PMID: 21537127 DOI: 10.1097/MEG.0b013e328346d505]

56 **Lewis AL**, Gonzalez MV, Leppard SW, Brown JE, Stratford PW, Phillips GJ, Lloyd AW. Doxorubicin eluting beads - 1: effects of drug loading on bead characteristics and drug distribution. *J Mater Sci Mater Med* 2007; **18**: 1691-1699 [PMID: 17483878 DOI: 10.1007/s10856-007-3068-8]

57 **Lesniak MS**, Upadhyay U, Goodwin R, Tyler B, Brem H. Local delivery of doxorubicin for the treatment of malignant brain tumors in rats. *Anticancer Res* 2005; **25**: 3825-3831 [PMID: 16312042]

58 **Salmaggi A**, Duri S, Silvani A, Gaviani P, Milanesi I, Casali C, Di Meco F. Loco-regional treatments in first-diagnosis glioblastoma: literature review on association between Stupp protocol and Gliadel. *Neurol Sci* 2011; **32** Suppl 2: S241-S245 [PMID: 21983866 DOI: 10.1007/s10072-011-0797-8]

59 **Hart MG**, Grant R, Garside R, Rogers G, Somerville M, Stein K. Chemotherapeutic wafers for High Grade Glioma. *Cochrane Database Syst Rev* 2008; (**3**): CD007294 [PMID: 18646178 DOI: 10.1002/14651858.CD007294]

60 **Baltes S**, Freund I, Lewis AL, Nolte I, Brinker T. Doxorubicin and irinotecan drug-eluting beads for treatment of glioma: a pilot study in a rat model. *J Mater Sci Mater Med* 2010; **21**: 1393-1402 [PMID: 20162337 DOI: 10.1007/s10856-009-3803-4]

61 **Noble CO**, Krauze MT, Drummond DC, Yamashita Y, Saito R, Berger MS, Kirpotin DB, Bankiewicz KS, Park JW. Novel nanoliposomal CPT-11 infused by convection-enhanced delivery in intracranial tumors: pharmacology and efficacy. *Cancer Res* 2006; **66**: 2801-2806 [PMID: 16510602 DOI: 10.1158/0008-5472.CAN-05-3535]

62 **Eyol E**, Boleij A, Taylor RR, Lewis AL, Berger MR. Chemoembolisation of rat colorectal liver metastases with drug eluting beads loaded with irinotecan or doxorubicin. *Clin Exp Metastasis* 2008; **25**: 273-282 [PMID: 18259882]

63 **Glage S**, Lewis AL, Mertens P, Baltes S, Geigle P, Brinker T. Evaluation of biocompatibility and anti-glioma efficacy of doxorubicin and irinotecan drug-eluting bead suspensions in alginate. *Clin Transl Oncol* 2012; **14**: 50-59 [PMID: 22262719 DOI: 10.1007/s12094-012-0761-y]

64 **Forster RE**, Small SA, Tang Y, Heaysman CL, Lloyd AW, Macfarlane W, Phillips GJ, Antonijevic MD, Lewis AL. Com-

parison of DC Bead-irinotecan and DC Bead-topotecan drug eluting beads for use in locoregional drug delivery to treat pancreatic cancer. *J Mater Sci Mater Med* 2010; **21**: 2683-2690 [PMID: 20563626 DOI: 10.1007/s10856-010-4107-4]

65 **Forster RE**, Tang Y, Bowyer C, Lloyd AW, Macfarlane W, Phillips GJ, Lewis AL. Development of a combination drug-eluting bead: towards enhanced efficacy for locoregional tumour therapies. *Anticancer Drugs* 2012; **23**: 355-369 [PMID: 22241169 DOI: 10.1097/CAD.0b013e32835006d2]

66 **Linghu E**, Matthes K, Mino-Kenudson M, Brugge WR. Feasibility of endoscopic ultrasound-guided OncoGel (ReGel/paclitaxel) injection into the pancreas in pigs. *Endoscopy* 2005; **37**: 1140-1142 [PMID: 16281147 DOI: 10.1055/s-2005-870224]

67 **Matthes K**, Mino-Kenudson M, Sahani DV, Holalkere N, Fowers KD, Rathi R, Brugge WR. EUS-guided injection of paclitaxel (OncoGel) provides therapeutic drug concentrations in the porcine pancreas (with video). *Gastrointest Endosc* 2007; **65**: 448-453 [PMID: 17173909 DOI: 10.1016/j.gie.2006.06.030]

68 **Karaca C**, Cizginer S, Konuk Y, Kambadakone A, Turner BG, Mino-Kenudson M, Sahani DV, Macfarlane C, Brugge W. Feasibility of EUS-guided injection of irinotecan-loaded microspheres into the swine pancreas. *Gastrointest Endosc* 2011; **73**: 603-606 [PMID: 21238959 DOI: 10.1016/j.gie.2010.11.003]

69 **Cerny T**, Martinelli G, Goldhirsch A, Terrier F, Joss R, Fey MF, Brunner KW, Küpfer A. Continuous 5-day infusion of ifosfamide with mesna in inoperable pancreatic cancer patients: a phase II study. *J Cancer Res Clin Oncol* 1991; **117 Suppl 4**: S135-S138 [PMID: 1795002]

70 **Keizer HJ**, Ouwerkerk J, Welvaart K, van der Velde CJ, Cleton FJ. Ifosfamide treatment as a 10-day continuous intravenous infusion. *J Cancer Res Clin Oncol* 1995; **121**: 297-302 [PMID: 7768968]

71 **Dirven HA**, van Ommen B, van Bladeren PJ. Glutathione conjugation of alkylating cytostatic drugs with a nitrogen mustard group and the role of glutathione S-transferases. *Chem Res Toxicol* 1996; **9**: 351-360 [PMID: 8839035 DOI: 10.1021/tx9500661]

72 **Zheng JJ**, Chan KK, Muggia F. Preclinical pharmacokinetics and stability of isophosphoramide mustard. *Cancer Chemother Pharmacol* 1994; **33**: 391-398 [PMID: 8306413]

73 **Löhr M**, Müller P, Karle P, Stange J, Mitzner S, Jesnowski R, Nizze H, Nebe B, Liebe S, Salmons B, Günzburg WH. Targeted chemotherapy by intratumour injection of encapsulated cells engineered to produce CYP2B1, an ifosfamide activating cytochrome P450. *Gene Ther* 1998; **5**: 1070-1078 [PMID: 10326030 DOI: 10.1038/sj.gt.3300671]

74 **Crandell RA**, Fabricant CG, Nelson-Rees WA. Development, characterization, and viral susceptibility of a feline (Felis catus) renal cell line (CRFK). *In Vitro* 1973; **9**: 176-185 [PMID: 4130570]

75 **Stange J**, Mitzner S, Dautzenberg H, Ramlow W, Knippel M, Steiner M, Ernst B, Schmidt R, Klinkmann H. Prolonged biochemical and morphological stability of encapsulated liver cells--a new method. *Biomater Artif Cells Immobilization Biotechnol* 1993; **21**: 343-352 [PMID: 8399975]

76 **Pear WS**, Nolan GP, Scott ML, Baltimore D. Production of high-titer helper-free retroviruses by transient transfection. *Proc Natl Acad Sci USA* 1993; **90**: 8392-8396 [PMID: 7690960]

77 **Takeuchi Y**, Porter CD, Strahan KM, Preece AF, Gustafsson K, Cosset FL, Weiss RA, Collins MK. Sensitization of cells and retroviruses to human serum by (alpha 1-3) galactosyltransferase. *Nature* 1996; **379**: 85-88 [PMID: 8538747 DOI: 10.1038/379085a0]

78 **Samel S**, Keese M, Lux A, Jesnowski R, Prosst R, Saller R, Hafner M, Sturm J, Post S, Löhr M. Peritoneal cancer treatment with CYP2B1 transfected, microencapsulated cells and ifosfamide. *Cancer Gene Ther* 2006; **13**: 65-73 [PMID: 16096652 DOI: 10.1038/sj.cgt.7700849]

79 **Sturm JW**, Keese MA, Petruch B, Bönninghoff RG, Zhang H, Gretz N, Hafner M, Post S, McCuskey RS. Enhanced green fluorescent protein-transfection of murine colon carcinoma cells: key for early tumor detection and quantification. *Clin Exp Metastasis* 2003; **20**: 395-405 [PMID: 14524528]

80 **Yagublu V**, Caliskan N, Lewis AL, Jesenofsky R, Gasimova L, Löhr JM, Keese M. Treatment of experimental pancreatic cancer by doxorubicin-, mitoxantrone-, and irinotecan-drug eluting beads. *Pancreatology* 2013; **13**: 79-87 [PMID: 23395574 DOI: 10.1016/j.pan.2012.11.305]

81 **Binder S**, Lewis AL, Jesenofsky R, Neumaier M, Jäger E, Ströbel P, Löhr JM, Keese M. Intraperitoneal use of doxorubicin drug-eluting beads in sheep: a pilot safety study. *Anticancer Res* 2012; **32**: 5167-5174 [PMID: 23225413]

82 **Ozols RF**, Young RC, Speyer JL, Sugarbaker PH, Greene R, Jenkins J, Myers CE. Phase I and pharmacological studies of adriamycin administered intraperitoneally to patients with ovarian cancer. *Cancer Res* 1982; **42**: 4265-4269 [PMID: 7105021]

83 **Abraham R**, Basser RL, Green MD. A risk-benefit assessment of anthracycline antibiotics in antineoplastic therapy. *Drug Saf* 1996; **15**: 406-429 [PMID: 8968695]

84 **Abu-Khalaf MM**, Harris L. Anthracycline-induced cardiotoxicity: risk assessment and management. *Oncology* (Williston Park) 2009; **23**: 239, 244, 252 [PMID: 19418825]

85 **Appel JM**, Nielsen D, Zerahn B, Jensen BV, Skagen K. Anthracycline-induced chronic cardiotoxicity and heart failure. *Acta Oncol* 2007; **46**: 576-580 [PMID: 17562433 DOI: 10.1080/02841860601156165]

86 **Klein HO**. Anthracycline-induced cardiotoxicity. *Ann Intern Med* 1997; **126**: 827; author reply 827-828 [PMID: 9148660]

87 **Shan K**, Lincoff AM, Young JB. Anthracycline-induced cardiotoxicity. *Ann Intern Med* 1996; **125**: 47-58 [PMID: 8644988]

88 **Ackland SP**, Ratain MJ, Vogelzang NJ, Choi KE, Ruane M, Sinkule JA. Pharmacokinetics and pharmacodynamics of long-term continuous-infusion doxorubicin. *Clin Pharmacol Ther* 1989; **45**: 340-347 [PMID: 2702792]

89 **Gottlieb JA**, Gutterman JU, McCredie KB, Rodriguez V, Frei E. Chemotherapy of malignant lymphoma with adriamycin. *Cancer Res* 1973; **33**: 3024-3028 [PMID: 4748450]

90 **Pfizer**. Adriblastin - Fachinformation. Berlin: Rote Liste Service GmbH, 2009

91 **Piscitelli SC**, Rodvold KA, Rushing DA, Tewksbury DA. Pharmacokinetics and pharmacodynamics of doxorubicin in patients with small cell lung cancer. *Clin Pharmacol Ther* 1993; **53**: 555-561 [PMID: 8387903]

92 **Litterst CL**, Collins JM, Lowe MC, Arnold ST, Powell DM, Guarino AM. Local and systemic toxicity resulting from large-volume ip administration of doxorubicin in the rat. *Cancer Treat Rep* 1982; **66**: 157-161 [PMID: 7053251]

93 **Schmoll HJ**. Kompendium internistische Onkologie. Standards in Diagnostik und Therapie: Springer; 1999

P- Reviewers: Ding MX, Filep JG, Tan XR **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.v19.i43.7594

World J Gastroenterol 2013 November 21; 19(43): 7594-7602
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Role of sirtuins in ischemia-reperfusion injury

Eirini Pantazi, Mohamed Amine Zaouali, Mohamed Bejaoui, Emma Folch-Puy, Hassen Ben Abdennebi, Joan Roselló-Catafau

Eirini Pantazi, Mohamed Amine Zaouali, Mohamed Bejaoui, Emma Folch-Puy, Experimental Hepatic Ischemia-Reperfusion Unit, Institut of Biomedical Research of Barcelona-Spanish National Research Council, Barcelona, 08036 Catalonia, Spain
Hassen Ben Abdennebi, Molecular Biology and Anthropology applied to development and health (UR12ES11), Faculty of Pharmacy, Monastir 5000, Tunisia
Joan Roselló-Catafau, Experimental Pathology Department, Institute of Biomedical Research of Barcelona-Spanish National Research Council, Barcelona, 08036 Catalonia, Spain
Author contributions: Pantazi E, Zaouali MA, Folch-Puy E and Bejaoui M wrote the article and reviewed the literature; Ben Abdennebi H and Roselló-Catafau J made significant contributions to the design and revision of the manuscript; all authors have read and approved the final manuscript.
Supported by AGAUR, No. 2012FI_B00382, Generalitat de Catalunya, Barcelona, Spain, to Pantazi E; CSIC for the development program to Bejaoui M, No. I-COOP0005; The Fondo de Investigaciones Sanitarias, No. FIS PI12/00519
Correspondence to: Joan Rosello-Catafau, PhD, Experimental Pathology Department, Institute of Biomedical Research of Barcelona-Spanish National Research Council, C/ Rosselló 161, 7th floor, Barcelona, 08036 Catalonia, Spain. jrcbam@iibb.csic.es
Telephone: +34-93-3638300 Fax: +34-93-3638301
Received: June 28, 2013 Revised: September 16, 2013
Accepted: September 29, 2013
Published online: November 21, 2013

Abstract

Ischemia-reperfusion injury (IRI) remains an unresolved and complicated situation in clinical practice, especially in the case of organ transplantation. Several factors contribute to its complexity; the depletion of energy during ischemia and the induction of oxidative stress during reperfusion initiate a cascade of pathways that lead to cell death and finally to severe organ injury. Recently, the sirtuin family of nicotinamide adenine dinucleotide-dependent deacetylases has gained increasing attention from researchers, due to their involvement in the modulation of a wide variety of cellular functions. There are seven mammalian sirtuins and, among them, the nuclear/cytoplasmic sirtuin 1 (SIRT1) and the mitochondrial sirtuin 3 (SIRT3) are ubiquitously expressed in many tissue types. Sirtuins are known to play major roles in protecting against cellular stress and in controlling metabolic pathways, which are key processes during IRI. In this review, we mainly focus on SIRT1 and SIRT3 and examine their role in modulating pathways against energy depletion during ischemia and their involvement in oxidative stress, apoptosis, microcirculatory stress and inflammation during reperfusion. We present evidence of the beneficial effects of sirtuins against IRI and emphasize the importance of developing new strategies by enhancing their action.

Key words: Sirtuin 1; Sirtuin 3; Ischemia-reperfusion injury; Oxidative stress; Apoptosis

Core tip: Sirtuins are responsible for the regulation of protein activation by deacetylating a range of proteins that play important roles in the pathophysiology of various diseases. The present review summarizes the beneficial effects of sirtuins 1 and 3, the two most prominent sirtuins involved in mammalian energy homeostasis and oxidative stress. We conclude that both sirtuins might be attractive targets for counteracting the detrimental effects of ischemia-reperfusion injury.

Pantazi E, Zaouali MA, Bejaoui M, Folch-Puy E, Ben Abdennebi H, Roselló-Catafau J. Role of sirtuins in ischemia-reperfusion injury. *World J Gastroenterol* 2013; 19(43): 7594-7602 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7594.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7594

INTRODUCTION

Sirtuins belong to the highly conserved class Ⅲ histone

deacetylases with homology to the yeast silent information regulator 2. To date, seven sirtuins have been described in mammals. They posses nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase activity, with the exception of sirtuin 4 (SIRT4) which has only ADP-ribosyltransferase activity, and SIRT1 and SIRT6 which have not only deacetylase activity but also relatively weak ADP-ribosyltransferase activity[1]. Their enzymatic activity depends on their protein expression levels, the availability of NAD^+ and the presence of proteins that modulate sirtuin enzymatic activity. For instance, SIRT1 expression increases during starvation or when cells are exposed to conditions of oxidative stress and DNA damage[2,3].

Sirtuins are found in several subcellular locations, including the nucleus (SIRT1, SIRT6, and SIRT7), cytosol (SIRT2), and mitochondria (SIRT3-SIRT5). In some studies, however, SIRT1 has been found to possess cytosolic activity, and SIRT2 has been found to be associated with nuclear proteins[4].

Several recent studies have shown that sirtuins regulate a wide variety of cellular processes, such as gene transcription, metabolism and cellular stress response[5-7]. SIRT1, the most studied member of the family, plays an important role in several processes ranging from cell cycle regulation to energy homeostasis[8,9]. SIRT3 has recently been reported to have a considerable impact on mitochondrial energy metabolism and function[10,11]. In this review, we will focus mainly on SIRT1 and SIRT3 functions in ischemia-reperfusion injury (IRI).

IRI is one of the most significant problems in graft injury, contributing to primary graft dysfunction or non-function after organ transplantation[12-14]. Many factors contribute to IRI. First of all, the loss of oxygen supply during ischemia results in the reduction of adenosine triphosphate (ATP) synthesis and subsequent changes in ion influx, acidosis and cell swelling which may eventually lead to cell death. The restoration of blood flow is followed by an excessive acute inflammatory response triggering the reperfusion injury. Although the ischemic insult causes significant damage in cells, the tissue injury generated during reperfusion is much more severe. On reperfusion, oxygen is suddenly available, and metabolism proceeds rapidly, resulting in a sudden production of reactive oxygen species (ROS), cytokines and chemokines which increase the accumulation of inflammatory cells (monocytes, dendritic cells and granulocytes). In combination with excessive nitric oxide (NO), ROS are able to induce DNA damage and activate various types of cell death pathways[15-17].

Understanding the mechanisms involved in the pathogenesis of IRI is the first step to mitigate its adverse effects. Sirtuins are known to regulate many important processes in cell physiology, including those affecting IRI, such as cellular metabolism and stress response. This makes them potentially appealing targets for therapeutic interventions against IR-induced injury.

ROLE OF SIRTUINS IN ISCHEMIA

The low energy state during ischemia results in activation of adenosine monophosphate protein kinase (AMPK), a fuel-sensing enzyme that is positively regulated by an increased ratio of adenosine monophosphate to ATP. When AMPK is activated, it stimulates processes that restore ATP levels (*e.g.*, fatty acid oxidation) and inhibits other processes that consume ATP (*e.g.*, protein synthesis)[18]. The activity of sirtuins is directly related to the metabolic state of the cell due to their dependence on NAD^+. Suchankova and collaborators found that glucose-induced changes in AMPK are linked to alterations in the NAD^+/reduced nicotinamide adenine dinucleotide ratio and SIRT1 abundance and activity[19]. These results may suggest a possible interaction between AMPK and SIRT1 in ischemic conditions. Indeed, an activator of AMPK, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside, has been found to improve IRI and increase SIRT1 expression in the rat kidney[20]. Furthermore, enhancing the activity of SIRT1 through the application of resveratrol, a SIRT1 activator, has been demonstrated to protect against cerebral ischemia[21].

Another element that plays an essential role in triggering cellular protection and preventing metabolic alterations caused by oxygen deprivation is hypoxia-inducible factors (HIFs). Mammals possess three isoforms of HIFα, of which HIF1α and HIF2α are the most structurally similar and the best characterized. During hypoxia, protein levels of HIF2α increase slightly, but it presents significant activation, which suggests that its activity is regulated by additional post-translational mechanisms. One of these post-translational modulations may be deacetylation, since in hypoxic Hep3B cells SIRT1 deacetylates lysine residues in the HIF2α protein, enhancing its transcriptional activity[22].

Additionally, SIRT1 interacts with HIF1α, but in this case SIRT1 represses HIF1α transcriptional activity[23]. Under hypoxic stress, decreased cellular NAD^+ downregulates SIRT1, increases HIF1α acetylation, and thereby promotes the expression of *HIF1α* target genes[23]. Interestingly, other studies have shown that HIF2α compete with HIF1α for binding to SIRT1[24]. Moreover, it has been demonstrated that SIRT6 is also linked to HIF1α by repressing the transcription of *HIF1α* target genes[25].

Likewise, the effects of SIRT3 appear to be protective in the context of hypoxic stress in human cancer cells. SIRT3 overexpression resulted in decreased ROS production, impediment of HIF1α stabilization and subsequent suppression of tumorigenesis[26,27]. However, the effect of SIRT3 in HIF1α stabilization in IRI has not been reported to date.

One of the most important factors involved in the metabolic control regulated by SIRT1 is peroxisome proliferator-activated receptor-γ coactivator 1α (PGC1α), a transcriptional co-activator of many nuclear receptors and transcriptional factors. SIRT1 functionally interacts with PGC1α and deacetylates it, thus inducing the expression of mitochondrial proteins involved in ATP-generating pathways[28]. Increased PGC1α activity is also associated with lower levels of oxidative damage during

ischemia, as shown by the decrease ROS scavenging in rodents lacking PGC1α subjected to global ischemia[29]. Furthermore, the uncoupling protein 2 (UCP2), an inner mitochondrial membrane protein, regulates the proton electrochemical gradient and in neuronal cells PGC1α is required for the induction of UCP2 and subsequent protection against oxidative stress[30]. It has also been shown that enhanced activity of SIRT1 during ischemic preconditioning (IPC) or resveratrol preconditioning confers protection against cerebral ischemia by reducing UCP2 levels, which results in increased ATP levels[21]. However, a more recent study associated the protective effect of resveratrol against oxidative stress in cerebral ischemia with increased levels of SIRT1/PGC1α and UCP2[31]. Moreover, the exact role of UCP2 during ischemia is not fully understood, as studies of its effects have produced conflicting results[32-35].

ROLE OF SIRTUINS IN REPERFUSION

Deprivation of oxygen to the grafts during ischemia induces severe lesions, but the most important damage is caused during reperfusion, when oxygen entry to the organ is restored. During reperfusion, the cellular metabolism returns to aerobic pathways, which results in the generation of a wide variety of ROS, including superoxide, hydrogen peroxide and reactive nitrogen species, such as peroxynitrite. ROS are mainly produced in mitochondria and trigger several phenomena, including accumulation of Ca^{2+}, caspase activation, cytokine upregulation, lipid, protein and DNA damage[36-38]. ROS can be eliminated by enzymatic pathways including manganese superoxide dismutase (MnSOD), catalase (Cat) and peroxidases. Imbalance between ROS generation and elimination produces oxidative stress[15,16].

Various reports in cardiomyocytes have demonstrated the protective role of SIRT1 against oxidative stress[39,40]. Hearts overexpressing SIRT1 were more resistant to oxidative stress in response to IRI, as SIRT1 upregulated the expression of anti-oxidants like MnSOD and thioredoxin 1[41]. SIRT1 also deacetylated Forkhead box-containing protein O (FoxO) 1 transcription factor, inducing its nuclear translocation and subsequent transcription of anti-oxidant molecules[41,42]. Moreover, the question of whether SIRT1 can induce the transcription of other FoxO transcription factors, like FoxO3α, has not yet been investigated. However, the levels of SIRT1 activation are decisive for its protective role, as very high cardiac SIRT1 expression induces mitochondrial dysfunction and increases oxidative stress[39]. Furthermore, in a model of kidney IRI, the protective effect of SIRT1 against oxidative stress has also been demonstrated since SIRT1 upregulated Cat levels and maintained peroxisome number and function[43].

Although mitochondrial sirtuins (SIRT3-SIRT5) have not been studied as extensively as SIRT1, an increasing body of evidence indicates the importance of SIRT3 in mitochondrial biology and function. Lombard *et al*[44] demonstrated that SIRT3 is the dominant mitochondrial deacetylase, as a significant number of mitochondrial proteins are hyperacetylated in $SIRT3^{-/-}$ mice. SIRT3 deacetylates and thus enhances the activity of various proteins that appear to be an important part of the anti-oxidative defense mechanisms of mitochondria, such as MnSOD[45,46], regulatory proteins of the glutathione[47-49] and thioredoxin system[50].

Transcriptional upregulation of the antioxidant enzymes MnSOD, Cat and peroxiredoxin can also be achieved by FoxO3α transcription factor, which is translocated to the nucleus after being deacetylated by SIRT3[51,52]. Furthermore, SIRT3 is necessary for the enhanced expression of cytochrome c, which presents peroxidase- and superoxidase-scavenging capacity[47,49,53]. However, a similar anti-oxidant effect of SIRT3 in models of IRI has not yet been established.

A wide array of functional alterations develop in mitochondria during reperfusion injury[36,54]. In healthy cells, their primary function is the provision of ATP through oxidative phosphorylation in order to meet the high energy demands. There is increasing evidence of the involvement of a multi-protein complex called the mitochondrial permeability transition pore (mPTP) in the decline in mitochondrial function, which is a common finding during reperfusion injury[55-57]. SIRT3 is known to deacetylate the regulatory component of the mPTP, cyclophilin D, and thereby reduce its activity and the subsequent mitochondrial swelling in the heart[58]. It has also been shown that SIRT4 interacts with the adenine nucleotide translocator, another component of mPTP, and that SIRT5 deacetylates cytochrome c, but the physiological importance of these interactions has not yet been established[59,60], especially in models of IRI.

Microcirculatory alterations play an important part in IRI. During the ischemic period, vascular hypoxia can cause increased vascular permeability. After reperfusion, complement system activation, leukocyte-endothelial cell adhesion and platelet-leukocyte aggregation further aggravate microvascular dysfunction[61].

NO produced by endothelial NO synthase (eNOS) is a key regulator of endothelial function, as it opposes the vasoconstrictive actions of endothelins and provokes vasodilatation. Thus, it can abrogate the microcirculatory stress generated during reperfusion[62]. However, NO produced by inducible NO synthase (iNOS) exacerbates IRI through the NOS-derived superoxide production or the generation of peroxynitrite[12]. There is a large body of evidence in favor of the relationship between eNOS and SIRT1; SIRT1 interacts and modifies the acetylation state of eNOS, resulting in the activation of the enzyme[63-65]. In $SIRT1^{+/+}$ hearts subjected to IRI SIRT1 was associated with eNOS activation[66]. SIRT1 activation by resveratrol protected against subacute intestinal IRI by reducing the NO production through iNOS[67] Moreover, various experimental models showed that resveratrol inhibits endothelin-1 levels, providing better regulation of vascular tone[68-70]. However, a recent study in human umbilical vein endothelial cells

has shown that the inhibitory effects of resveratrol on endothelin-1 levels are SIRT1-independent[71].

ROLE OF SIRTUINS IN IRI-ASSOCIATED INFLAMMATION

IRI results in a profound inflammatory tissue reaction with immune cells infiltrating the tissue. The damage is mediated by various cytokines, chemokines, adhesion molecules, and compounds of the extracellular matrix. The expression of these factors is regulated by specific transcription factors with nuclear factor kappa B (NF-κB) being one of the key modulators of inflammation. After activation, the transcription factor migrates to the nucleus and enhances the transcription of pro-inflammatory genes potentiating the inflammatory response. This is followed by an infiltration of lymphocytes, mononuclear cells/macrophages, and granulocytes into the injured tissue[72-74].

In this way, SIRT1 plays an important role in neuroprotection against brain ischemia by deacetylation and subsequent inhibition of p53 and NF-κB pathways[75]. In $SIRT1^{+/+}$ hearts subjected to IRI SIRT1 was correlated with decreased acetylation of NF-κB and possible prevention of inflammation[66]. Moreover, the anti-inflammatory action of SIRT1 by deacetylating NF-κB and thus inhibiting the expression of endothelial adhesion molecules has also been demonstrated in human aortic endothelial cells[74].

SIRTUINS: CELL SURVIVAL OR DEATH?

Apoptotic cell death is a well known mechanism involved in IRI which occurs *via* activation of caspases that cleave DNA and other cellular components[16,17,76]. There is evidence that SIRT1 is associated with longevity in mammals and enhances mammalian cell survival under stress conditions *via* regulating the specific substrates[77-79]. In fact, several studies have mentioned the anti-apoptotic effect of SIRT1 in IRI. SIRT1 deacetylates known mediators of apoptosis, such as the tumor-suppressor p53, resulting in inhibition of its transcriptional activity[80,81]. SIRT1 also deacetylates the DNA repair factor Ku70[2,82,83]; thus Ku70 prevents the translocation of Bax, a pro-apoptotic B cell lymphoma-2 (Bcl-2) family protein, to the mitochondria. In ischemic kidney and brain SIRT1 has been identified as an important survival mediator, given that increased SIRT1 was associated with reduced p53 expression and apoptosis[75,84]. SIRT1 also modulates apoptosis-related molecules through the deacetylation of the FoxO family of transcription factors. During IRI in heart-specific $SIRT1^{+/+}$ transgenic mice, SIRT1 induces nuclear translocation of FoxO1, which upregulates the anti-apoptotic factors Bcl-2 and Bcl-like X and downregulates Bax[41]. As regards other members of the FoxO family, Brunet *et al*[85] revealed a dual role of SIRT1 in the cell cycle depending on stress conditions; SIRT1 inhibited the ability of FoxO3 to induce cell death, thus promoting cell survival and, surprisingly, it also increased the ability of FoxO3 to induce cell cycle arrest and resistance to oxidative stress.

A possible pro-apoptotic role of SIRT1 in IRI has not been reported previously. However, studies in human embryonic kidney cells have revealed that SIRT1 can promote cell death by inhibiting NF-κB in response to tumor necrosis factor alpha[86]. Further investigation is required to define the conditions under which SIRT1 may promote apoptosis.

Apoptotic pathways are known to be initiated during reperfusion upon the opening of the mPTP which leads to the release of caspase-activating molecules[87,88]. Since SIRT3 is located in the mitochondria, it may be involved in anti-apoptotic pathways. In this regard, SIRT3 protects various types of cells from apoptotic cell death triggered by genotoxic or oxidative stress[89-92]. The pro-apoptotic role of SIRT3 has also been associated with tumor suppression and restraint of ROS[93]. However, SIRT3 has also been reported to contribute to Bcl-2- and JNK-related apoptotic pathways in human colorectal carcinoma cells[94]. In any case, the potential anti-apoptotic mechanisms of SIRT3 during IRI are yet to be elucidated.

CONCLUSIONS AND PERSPECTIVES

A wide range of pathological processes contribute to IRI. Particularly during organ transplantation, IRI contributes to early graft dysfunction. For this reason, it is important to gain additional mechanistic insight into the molecular mechanisms underlying this injury. In the past few years, sirtuins have emerged as critical modulators of various cellular processes, including those that contribute to the pathogenesis of IRI.

In this paper, we have reviewed the signaling pathways of SIRT1 and SIRT3 protection in IRI. SIRT1 has been shown to exert its beneficial effect against oxidative stress, hypoxic injury or inflammation associated with IRI by activating FoxO1, PGC1α and HIF2α or by inhibiting NF-κB transcription factors (Figures 1 and 2). SIRT3's protective role in IRI is mainly mediated by activating FoxO3α and mitochondrial anti-oxidant enzymes (Figure 2). Investigations that can further determine other intracellular signaling, trafficking and post-translational modifications by SIRT1 and SIRT3 in a variety of cell systems and environments will allow us to translate this knowledge into effective treatment strategies that will be applicable in multiple disorders.

Numerous studies have demonstrated key roles for SIRT1 and SIRT3 in brain, heart and kidney IRI. However, the protective effect of these sirtuins against ischemic processes in other organs such as the liver has not yet been demonstrated. The relevance of SIRT3 in the hepatic metabolism has been confirmed in a study showing that its overexpression in hepatocytes decreased the accumulation of lipids *via* AMPK activation[95]. Furthermore, deletion of hepatic SIRT1 resulted in hepatic steatosis, hepatic inflammation and endoplasmatic reticulum stress[96]. Since SIRT1 and SIRT3 have been shown

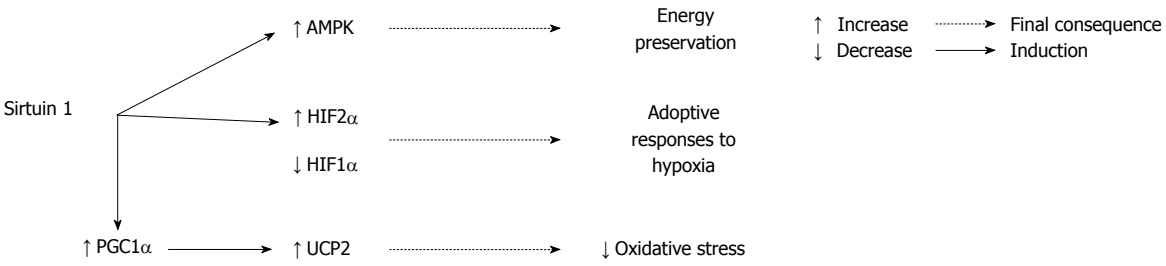


Figure 1 Protective role of sirtuin 1 during ischemia. Sirtuin 1 (SIRT1) activates adenosine monophosphate protein kinase (AMPK) as a cell response to counteract the energy deficiency. SIRT1 upregulates hypoxia-inducible factor 2α (HIF2α) and downregulates HIF1α to increase their transcriptional activity. SIRT1 upregulates peroxisome proliferator-activated receptor-γ coactivator, leading to enhancement of anti-oxidant capacity of uncoupling protein 2 (UCP2). PGC1α: Peroxisome proliferator-activated receptor-γ coactivator.

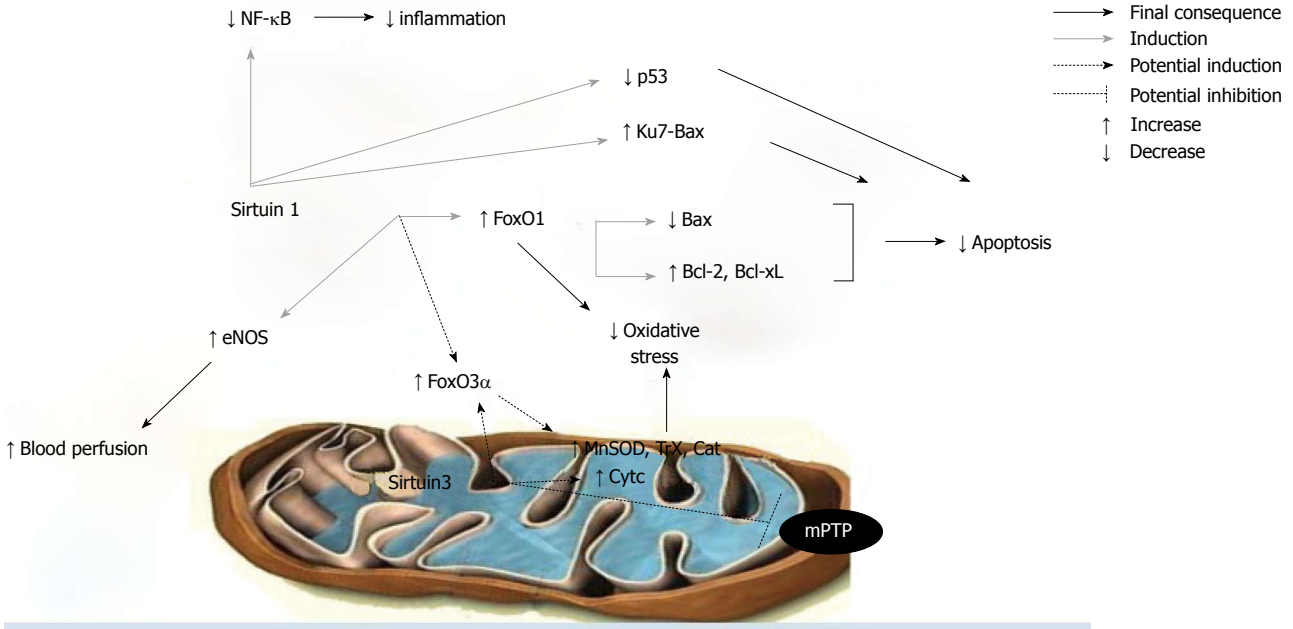


Figure 2 Protective role of sirtuin 1 and suggestive role of sirtuin 3 during reperfusion. Sirtuin 1 (SIRT1) inhibits inflammation through inhibition of nuclear factor kappa B and activates endothelial nitric oxide synthase for a better microcirculation. SIRT1 downregulates apoptosis through multiple pathways, for example, inhibiting p53 transcriptional activity or favoring the binding between Ku70 and Bax. SIRT1 also enhances forkhead box-containing protein O 1 (FoxO1) transcriptional activity, resulting in Bax downregulation and in the upregulation of B cell lymphoma-2 and Bcl-like X. Deacetylation of FoxO1 by SIRT1 also results in lessening oxidative stress, whereas the same effect may be achieved by deacetylation of forkhead box-containing protein 3 alpha (FoxO3α). Sirtuin 3 (SIRT3) is suggested to contribute to decrease in oxidative stress either by a direct interaction with mitochondrial anti-oxidant enzymes [manganese superoxide dismutase (MnSOD), thioredoxin system (Trx), cytochrome (Cyt)] or by enhancing FoxO3α to transcribe MnSOD and Cat. Mitochondrial permeability transition pore (mPTP) may also be inhibited by SIRT3 and result in less production of oxidative stress. NF-κB: Nuclear factor kappa B; eNOS: Endothelial nitric oxide synthase; Bcl-2: B cell lymphoma-2; Bcl-xL: Bcl-like X; Bax: Bcl-2-associated X; Cat: Catalase.

to exert a beneficial effect in regulating hepatic fatty acid metabolism, it would be interesting to investigate their role in the context of liver transplantation. Currently, the shortage of organs for transplantation has obliged physicians to utilize marginal grafts, including grafts with moderate steatosis. Steatotic livers exhibit a more severe inflammatory reaction and more exacerbated oxidative stress and consequently a higher vulnerability to IRI[12]. Thus, activating SIRT1 and SIRT3 might be a potential strategy to protect steatotic livers from IRI as well as to expand the donor pool for liver transplantation. In fact, in preliminary studies our group observed that SIRT1 is involved in the protective mechanisms against IRI elicited by IPC in fatty livers.

For this reason, both surgical and pharmacological strategies should be developed to enhance the activity of sirtuins and thus mitigate the detrimental effect of IRI. Recent studies have highlighted the important role of SIRT1 in IPC-mediated protection in the heart and brain; in IPC brain, SIRT1 prevents neuronal death[97], whereas during cardiac IPC, SIRT1 regulates HIF1α protein levels[98,99]. A recent review has also associated SIRT1 with the protective effects of hyperbaric oxygen preconditioning against apoptosis in the rat brain[100]. However, it is still to be established whether SIRT1 contributes to the protective effects of preconditioning through the regulation of other signalling pathways. Furthermore, its possible implication in IPC related mechanisms in other organs, including the liver or kidney, remains to be elucidated.

Nor has the potential role of sirtuins in cold ischemia

and reperfusion yet been established. In the context of liver IRI, a previous study by our group demonstrated that during normoxic reperfusion, after cold ischemia, the presence of NO favors HIF1α accumulation, also promoting the activation of other cytoprotective proteins, such as heme oxygenase-1[101]. Among these cytoprotective proteins, SIRT1 may be ideally suited to enhance the protective effect.

This review summarizes the basic mediators of IRI influenced by the action of SIRT1 and SIRT3 and highlights the importance of their regulation. Future research should aim to elucidate the complete action of all members of the sirtuins family in IRI, and to develop pharmacological strategies that can allow their action to be modulated.

REFERENCES

1 **Nakagawa T**, Guarente L. Sirtuins at a glance. *J Cell Sci* 2011; **124**: 833-838 [PMID: 21378304 DOI: 10.1242/jcs.081067]

2 **Cohen HY**, Miller C, Bitterman KJ, Wall NR, Hekking B, Kessler B, Howitz KT, Gorospe M, de Cabo R, Sinclair DA. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science* 2004; **305**: 390-392 [PMID: 15205477 DOI: 10.1126/science.1099196]

3 **Rodgers JT**, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P. Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. *Nature* 2005; **434**: 113-118 [PMID: 15744310 DOI: 10.1038/nature03354]

4 **Morris KC**, Lin HW, Thompson JW, Perez-Pinzon MA. Pathways for ischemic cytoprotection: role of sirtuins in caloric restriction, resveratrol, and ischemic preconditioning. *J Cereb Blood Flow Metab* 2011; **31**: 1003-1019 [PMID: 21224864 DOI: 10.1038/jcbfm.2010.229]

5 **Nogueiras R**, Habegger KM, Chaudhary N, Finan B, Banks AS, Dietrich MO, Horvath TL, Sinclair DA, Pfluger PT, Tschöp MH. Sirtuin 1 and sirtuin 3: physiological modulators of metabolism. *Physiol Rev* 2012; **92**: 1479-1514 [PMID: 22811431 DOI: 10.1152/physrev.00022.2011]

6 **Pillarisetti S**. A review of Sirt1 and Sirt1 modulators in cardiovascular and metabolic diseases. *Recent Pat Cardiovasc Drug Discov* 2008; **3**: 156-164 [PMID: 18991791]

7 **Gorenne I**, Kumar S, Gray K, Figg N, Yu H, Mercer J, Bennett M. Vascular smooth muscle cell sirtuin 1 protects against DNA damage and inhibits atherosclerosis. *Circulation* 2013; **127**: 386-396 [PMID: 23224247 DOI: 10.1161/CIRCULATIONAHA.112.124404]

8 **Al Massadi O**, Quiñones M, Lear P, Dieguez C, Nogueiras R. The Brain: A New Organ for the Metabolic Actions of SIRT1. *Horm Metab Res* 2013 Aug 15; Epub ahead of print [PMID: 23950036 DOI: 10.1055/s-0033-1351322]

9 **Gillum MP**, Kotas ME, Erion DM, Kursawe R, Chatterjee P, Nead KT, Muise ES, Hsiao JJ, Frederick DW, Yonemitsu S, Banks AS, Qiang L, Bhanot S, Olefsky JM, Sears DD, Caprio S, Shulman GI. SirT1 regulates adipose tissue inflammation. *Diabetes* 2011; **60**: 3235-3245 [PMID: 22110092 DOI: 10.2337/db11-0616]

10 **Brenmoehl J**, Hoeflich A. Dual control of mitochondrial biogenesis by sirtuin 1 and sirtuin 3. *Mitochondrion* 2013 Apr 11; Epub ahead of print [PMID: 23583953 DOI: 10.1016/j.mito.2013.04.002]

11 **Rardin MJ**, Newman JC, Held JM, Cusack MP, Sorensen DJ, Li B, Schilling B, Mooney SD, Kahn CR, Verdin E, Gibson BW. Label-free quantitative proteomics of the lysine acetylome in mitochondria identifies substrates of SIRT3 in metabolic pathways. *Proc Natl Acad Sci USA* 2013; **110**: 6601-6606 [PMID: 23576753 DOI: 10.1073/pnas.1302961110]

12 **Casillas-Ramírez A**, Mosbah IB, Ramalho F, Roselló-Catafau J, Peralta C. Past and future approaches to ischemia-reperfusion lesion associated with liver transplantation. *Life Sci* 2006; **79**: 1881-1894 [PMID: 16828807 DOI: 10.1016/j.lfs.2006.06.024]

13 **Treska V**, Kobr J, Hasman D, Racek J, Trefil L, Reischig T, Hes O, Kuntscher V, Molacek J, Treska I. Ischemia-reperfusion injury in kidney transplantation from non-heart-beating donor--do antioxidants or antiinflammatory drugs play any role? *Bratisl Lek Listy* 2009; **110**: 133-136 [PMID: 19507631]

14 **Schemmer P**, Lemasters JJ, Clavien PA. Ischemia/Reperfusion injury in liver surgery and transplantation. *HPB Surg* 2012; **2012**: 453295 [PMID: 23345924 DOI: 10.1155/2012/453295]

15 **Czubkowski P**, Socha P, Pawlowska J. Oxidative stress in liver transplant recipients. *Ann Transplant* 2011; **16**: 99-108 [PMID: 21436783]

16 **Datta G**, Fuller BJ, Davidson BR. Molecular mechanisms of liver ischemia reperfusion injury: insights from transgenic knockout models. *World J Gastroenterol* 2013; **19**: 1683-1698 [PMID: 23555157 DOI: 10.3748/wjg.v19.i11.1683]

17 **Murphy E**, Steenbergen C. Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. *Physiol Rev* 2008; **88**: 581-609 [PMID: 18391174 DOI: 10.1152/physrev.00024.2007]

18 **Ruderman NB**, Xu XJ, Nelson L, Cacicedo JM, Saha AK, Lan F, Ido Y. AMPK and SIRT1: a long-standing partnership? *Am J Physiol Endocrinol Metab* 2010; **298**: E751-E760 [PMID: 20103737 DOI: 10.1152/ajpendo.00745.2009]

19 **Suchankova G**, Nelson LE, Gerhart-Hines Z, Kelly M, Gauthier MS, Saha AK, Ido Y, Puigserver P, Ruderman NB. Concurrent regulation of AMP-activated protein kinase and SIRT1 in mammalian cells. *Biochem Biophys Res Commun* 2009; **378**: 836-841 [PMID: 19071085 DOI: 10.1016/j.bbrc.2008.11.130]

20 **Lempiäinen J**, Finckenberg P, Levijoki J, Mervaala E. AMPK activator AICAR ameliorates ischaemia reperfusion injury in the rat kidney. *Br J Pharmacol* 2012; **166**: 1905-1915 [PMID: 22324445 DOI: 10.1111/j.1476-5381.2012.01895.x]

21 **Della-Morte D**, Dave KR, DeFazio RA, Bao YC, Raval AP, Perez-Pinzon MA. Resveratrol pretreatment protects rat brain from cerebral ischemic damage via a sirtuin 1-uncoupling protein 2 pathway. *Neuroscience* 2009; **159**: 993-1002 [PMID: 19356683 DOI: 10.1016/j.neuroscience.2009.01.017]

22 **Dioum EM**, Chen R, Alexander MS, Zhang Q, Hogg RT, Gerard RD, Garcia JA. Regulation of hypoxia-inducible factor 2alpha signaling by the stress-responsive deacetylase sirtuin 1. *Science* 2009; **324**: 1289-1293 [PMID: 19498162 DOI: 10.1126/science.1169956]

23 **Lim JH**, Lee YM, Chun YS, Chen J, Kim JE, Park JW. Sirtuin 1 modulates cellular responses to hypoxia by deacetylating hypoxia-inducible factor 1alpha. *Mol Cell* 2010; **38**: 864-878 [PMID: 20620956 DOI: 10.1016/j.molcel.2010.05.023]

24 **Majmundar AJ**, Wong WJ, Simon MC. Hypoxia-inducible factors and the response to hypoxic stress. *Mol Cell* 2010; **40**: 294-309 [PMID: 20965423 DOI: 10.1016/j.molcel.2010.09.022]

25 **Zhong L**, D'Urso A, Toiber D, Sebastian C, Henry RE, Vadysirisack DD, Guimaraes A, Marinelli B, Wikstrom JD, Nir T, Clish CB, Vaitheesvaran B, Iliopoulos O, Kurland I, Dor Y, Weissleder R, Shirihai OS, Ellisen LW, Espinosa JM, Mostoslavsky R. The histone deacetylase Sirt6 regulates glucose homeostasis via Hif1alpha. *Cell* 2010; **140**: 280-293 [PMID: 20141841 DOI: 10.1016/j.cell.2009.12.041]

26 **Bell EL**, Emerling BM, Ricoult SJ, Guarente L. SirT3 suppresses hypoxia inducible factor 1α and tumor growth by inhibiting mitochondrial ROS production. *Oncogene* 2011; **30**: 2986-2996 [PMID: 21358671 DOI: 10.1038/onc.2011.37]

27 **Finley LW**, Carracedo A, Lee J, Souza A, Egia A, Zhang J, Teruya-Feldstein J, Moreira PI, Cardoso SM, Clish CB, Pandolfi PP, Haigis MC. SIRT3 opposes reprogramming

of cancer cell metabolism through HIF1α destabilization. *Cancer Cell* 2011; **19**: 416-428 [PMID: 21397863 DOI: 10.1016/j.ccr.2011.02.014]

28 **Nemoto S**, Fergusson MM, Finkel T. SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1{alpha}. *J Biol Chem* 2005; **280**: 16456-16460 [PMID: 15716268 DOI: 10.1074/jbc.M501485200]

29 **Chen SD**, Lin TK, Yang DI, Lee SY, Shaw FZ, Liou CW, Chuang YC. Protective effects of peroxisome proliferator-activated receptors gamma coactivator-1alpha against neuronal cell death in the hippocampal CA1 subfield after transient global ischemia. *J Neurosci Res* 2010; **88**: 605-613 [PMID: 19774674 DOI: 10.1002/jnr.22225]

30 **St-Pierre J**, Drori S, Uldry M, Silvaggi JM, Rhee J, Jäger S, Handschin C, Zheng K, Lin J, Yang W, Simon DK, Bachoo R, Spiegelman BM. Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell* 2006; **127**: 397-408 [PMID: 17055439 DOI: 10.1016/j.cell.2006.09.024]

31 **Shin JA**, Lee KE, Kim HS, Park EM. Acute resveratrol treatment modulates multiple signaling pathways in the ischemic brain. *Neurochem Res* 2012; **37**: 2686-2696 [PMID: 22878646 DOI: 10.1007/s11064-012-0858-2]

32 **Mattiasson G**, Shamloo M, Gido G, Mathi K, Tomasevic G, Yi S, Warden CH, Castilho RF, Melcher T, Gonzalez-Zulueta M, Nikolich K, Wieloch T. Uncoupling protein-2 prevents neuronal death and diminishes brain dysfunction after stroke and brain trauma. *Nat Med* 2003; **9**: 1062-1068 [PMID: 12858170 DOI: 10.1038/nm903]

33 **McLeod CJ**, Aziz A, Hoyt RF, McCoy JP, Sack MN. Uncoupling proteins 2 and 3 function in concert to augment tolerance to cardiac ischemia. *J Biol Chem* 2005; **280**: 33470-33476 [PMID: 16079144 DOI: 10.1074/jbc.M505258200]

34 **de Bilbao F**, Arsenijevic D, Vallet P, Hjelle OP, Ottersen OP, Bouras C, Raffin Y, Abou K, Langhans W, Collins S, Plamondon J, Alves-Guerra MC, Haguenauer A, Garcia I, Richard D, Ricquier D, Giannakopoulos P. Resistance to cerebral ischemic injury in UCP2 knockout mice: evidence for a role of UCP2 as a regulator of mitochondrial glutathione levels. *J Neurochem* 2004; **89**: 1283-1292 [PMID: 15147521 DOI: 10.1111/j.1471-4159.2004.02432.x]

35 **Bodyak N**, Rigor DL, Chen YS, Han Y, Bisping E, Pu WT, Kang PM. Uncoupling protein 2 modulates cell viability in adult rat cardiomyocytes. *Am J Physiol Heart Circ Physiol* 2007; **293**: H829-H835 [PMID: 17468330 DOI: 10.1152/ajpheart.01409.2006]

36 **Sanderson TH**, Reynolds CA, Kumar R, Przyklenk K, Hüttemann M. Molecular mechanisms of ischemia-reperfusion injury in brain: pivotal role of the mitochondrial membrane potential in reactive oxygen species generation. *Mol Neurobiol* 2013; **47**: 9-23 [PMID: 23011809 DOI: 10.1007/s12035-012-8344-z]

37 **Rodrigo R**, Prieto JC, Castillo R. Cardioprotection against ischaemia/reperfusion by vitamins C and E plus n-3 fatty acids: molecular mechanisms and potential clinical applications. *Clin Sci* (Lond) 2013; **124**: 1-15 [PMID: 22963444 DOI: 10.1042/CS20110663]

38 **Raedschelders K**, Ansley DM, Chen DD. The cellular and molecular origin of reactive oxygen species generation during myocardial ischemia and reperfusion. *Pharmacol Ther* 2012; **133**: 230-255 [PMID: 22138603 DOI: 10.1016/j.pharmthera.2011.11.004]

39 **Alcendor RR**, Gao S, Zhai P, Zablocki D, Holle E, Yu X, Tian B, Wagner T, Vatner SF, Sadoshima J. Sirt1 regulates aging and resistance to oxidative stress in the heart. *Circ Res* 2007; **100**: 1512-1521 [PMID: 17446436 DOI: 10.1161/01.RES.0000267723.65696.4a]

40 **Vinciguerra M**, Santini MP, Martinez C, Pazienza V, Claycomb WC, Giuliani A, Rosenthal N. mIGF-1/JNK1/SirT1 signaling confers protection against oxidative stress in the heart. *Aging Cell* 2012; **11**: 139-149 [PMID: 22051242 DOI: 10.1111/j.1474-9726.2011.00766.x]

41 **Hsu CP**, Zhai P, Yamamoto T, Maejima Y, Matsushima S, Hariharan N, Shao D, Takagi H, Oka S, Sadoshima J. Silent information regulator 1 protects the heart from ischemia/reperfusion. *Circulation* 2010; **122**: 2170-2182 [PMID: 21060073 DOI: 10.1161/CIRCULATIONAHA.110.958033]

42 **Daitoku H**, Hatta M, Matsuzaki H, Aratani S, Ohshima T, Miyagishi M, Nakajima T, Fukamizu A. Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity. *Proc Natl Acad Sci USA* 2004; **101**: 10042-10047 [PMID: 15220471 DOI: 10.1073/pnas.0400593101]

43 **Hasegawa K**, Wakino S, Yoshioka K, Tatematsu S, Hara Y, Minakuchi H, Sueyasu K, Washida N, Tokuyama H, Tzukerman M, Skorecki K, Hayashi K, Itoh H. Kidney-specific overexpression of Sirt1 protects against acute kidney injury by retaining peroxisome function. *J Biol Chem* 2010; **285**: 13045-13056 [PMID: 20139070 DOI: 10.1074/jbc.M109.067728]

44 **Lombard DB**, Alt FW, Cheng HL, Bunkenborg J, Streeper RS, Mostoslavsky R, Kim J, Yancopoulos G, Valenzuela D, Murphy A, Yang Y, Chen Y, Hirschey MD, Bronson RT, Haigis M, Guarente LP, Farese RV, Weissman S, Verdin E, Schwer B. Mammalian Sir2 homolog SIRT3 regulates global mitochondrial lysine acetylation. *Mol Cell Biol* 2007; **27**: 8807-8814 [PMID: 17923681 DOI: 10.1128/MCB.01636-07]

45 **Chen Y**, Zhang J, Lin Y, Lei Q, Guan KL, Zhao S, Xiong Y. Tumour suppressor SIRT3 deacetylates and activates manganese superoxide dismutase to scavenge ROS. *EMBO Rep* 2011; **12**: 534-541 [PMID: 21566644 DOI: 10.1038/embor.2011.65]

46 **Qiu X**, Brown K, Hirschey MD, Verdin E, Chen D. Calorie restriction reduces oxidative stress by SIRT3-mediated SOD2 activation. *Cell Metab* 2010; **12**: 662-667 [PMID: 21109198 DOI: 10.1016/j.cmet.2010.11.015]

47 **Zhao S**, Xu W, Jiang W, Yu W, Lin Y, Zhang T, Yao J, Zhou L, Zeng Y, Li H, Li Y, Shi J, An W, Hancock SM, He F, Qin L, Chin J, Yang P, Chen X, Lei Q, Xiong Y, Guan KL. Regulation of cellular metabolism by protein lysine acetylation. *Science* 2010; **327**: 1000-1004 [PMID: 20167786 DOI: 10.1126/science.1179689]

48 **Someya S**, Yu W, Hallows WC, Xu J, Vann JM, Leeuwenburgh C, Tanokura M, Denu JM, Prolla TA. Sirt3 mediates reduction of oxidative damage and prevention of age-related hearing loss under caloric restriction. *Cell* 2010; **143**: 802-812 [PMID: 21094524 DOI: 10.1016/j.cell.2010.10.002]

49 **Choudhary C**, Kumar C, Gnad F, Nielsen ML, Rehman M, Walther TC, Olsen JV, Mann M. Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science* 2009; **325**: 834-840 [PMID: 19608861 DOI: 10.1126/science.1175371]

50 **Palacios OM**, Carmona JJ, Michan S, Chen KY, Manabe Y, Ward JL, Goodyear LJ, Tong Q. Diet and exercise signals regulate SIRT3 and activate AMPK and PGC-1alpha in skeletal muscle. *Aging* (Albany NY) 2009; **1**: 771-783 [PMID: 20157566]

51 **Sundaresan NR**, Gupta M, Kim G, Rajamohan SB, Isbatan A, Gupta MP. Sirt3 blocks the cardiac hypertrophic response by augmenting Foxo3a-dependent antioxidant defense mechanisms in mice. *J Clin Invest* 2009; **119**: 2758-2771 [PMID: 19652361 DOI: 10.1172/JCI39162]

52 **Jacobs KM**, Pennington JD, Bisht KS, Aykin-Burns N, Kim HS, Mishra M, Sun L, Nguyen P, Ahn BH, Leclerc J, Deng CX, Spitz DR, Gius D. SIRT3 interacts with the daf-16 homolog FOXO3a in the mitochondria, as well as increases FOXO3a dependent gene expression. *Int J Biol Sci* 2008; **4**: 291-299 [PMID: 18781224]

53 **Kong X**, Wang R, Xue Y, Liu X, Zhang H, Chen Y, Fang F, Chang Y. Sirtuin 3, a new target of PGC-1alpha, plays an important role in the suppression of ROS and mitochondrial

biogenesis. *PLoS One* 2010; **5**: e11707 [PMID: 20661474 DOI: 10.1371/journal.pone.0011707]
54 **Andrews DT**, Royse C, Royse AG. The mitochondrial permeability transition pore and its role in anaesthesia-triggered cellular protection during ischaemia-reperfusion injury. *Anaesth Intensive Care* 2012; **40**: 46-70 [PMID: 22313063]
55 **Halestrap AP**. Calcium, mitochondria and reperfusion injury: a pore way to die. *Biochem Soc Trans* 2006; **34**: 232-237 [PMID: 16545083 DOI: 10.1042/BST20060232]
56 **Halestrap AP**, Clarke SJ, Javadov SA. Mitochondrial permeability transition pore opening during myocardial reperfusion--a target for cardioprotection. *Cardiovasc Res* 2004; **61**: 372-385 [PMID: 14962470 DOI: 10.1016/S0008-6363(03)00533-9]
57 **Shahzad T**, Kasseckert SA, Iraqi W, Johnson V, Schulz R, Schlüter KD, Dörr O, Parahuleva M, Hamm C, Ladilov Y, Abdallah Y. Mechanisms involved in postconditioning protection of cardiomyocytes against acute reperfusion injury. *J Mol Cell Cardiol* 2013; **58**: 209-216 [PMID: 23328483 DOI: 10.1016/j.yjmcc.2013.01.003]
58 **Hafner AV**, Dai J, Gomes AP, Xiao CY, Palmeira CM, Rosenzweig A, Sinclair DA. Regulation of the mPTP by SIRT3-mediated deacetylation of CypD at lysine 166 suppresses age-related cardiac hypertrophy. *Aging* (Albany NY) 2010; **2**: 914-923 [PMID: 21212461]
59 **Ahuja N**, Schwer B, Carobbio S, Waltregny D, North BJ, Castronovo V, Maechler P, Verdin E. Regulation of insulin secretion by SIRT4, a mitochondrial ADP-ribosyltransferase. *J Biol Chem* 2007; **282**: 33583-33592 [PMID: 17715127 DOI: 10.1074/jbc.M705488200]
60 **Schlicker C**, Gertz M, Papatheodorou P, Kachholz B, Becker CF, Steegborn C. Substrates and regulation mechanisms for the human mitochondrial sirtuins Sirt3 and Sirt5. *J Mol Biol* 2008; **382**: 790-801 [PMID: 18680753 DOI: 10.1016/j.jmb.2008.07.048]
61 **Eltzschig HK**, Collard CD. Vascular ischaemia and reperfusion injury. *Br Med Bull* 2004; **70**: 71-86 [PMID: 15494470 DOI: 10.1093/bmb/ldh025]
62 **Schmitt CA**, Heiss EH, Dirsch VM. Effect of resveratrol on endothelial cell function: Molecular mechanisms. *Biofactors* 2010; **36**: 342-349 [PMID: 20730905 DOI: 10.1002/biof.109]
63 **Ota H**, Eto M, Ogawa S, Iijima K, Akishita M, Ouchi Y. SIRT1/eNOS axis as a potential target against vascular senescence, dysfunction and atherosclerosis. *J Atheroscler Thromb* 2010; **17**: 431-435 [PMID: 20215708]
64 **Donato AJ**, Magerko KA, Lawson BR, Durrant JR, Lesniewski LA, Seals DR. SIRT-1 and vascular endothelial dysfunction with ageing in mice and humans. *J Physiol* 2011; **589**: 4545-4554 [PMID: 21746786 DOI: 10.1113/jphysiol.2011.211219]
65 **Mattagajasingh I**, Kim CS, Naqvi A, Yamamori T, Hoffman TA, Jung SB, DeRicco J, Kasuno K, Irani K. SIRT1 promotes endothelium-dependent vascular relaxation by activating endothelial nitric oxide synthase. *Proc Natl Acad Sci USA* 2007; **104**: 14855-14860 [PMID: 17785417 DOI: 10.1073/pnas.0704329104]
66 **Nadtochiy SM**, Yao H, McBurney MW, Gu W, Guarente L, Rahman I, Brookes PS. SIRT1-mediated acute cardioprotection. *Am J Physiol Heart Circ Physiol* 2011; **301**: H1506-H1512 [PMID: 21856913 DOI: 10.1152/ajpheart.00587.2011]
67 **Dong W**, Li F, Pan Z, Liu S, Yu H, Wang X, Bi S, Zhang W. Resveratrol ameliorates subacute intestinal ischemia-reperfusion injury. *J Surg Res* 2013; **185**: 182-189 [PMID: 23735732 DOI: 10.1016/j.jss.2013.05.013]
68 **Liu JC**, Chen JJ, Chan P, Cheng CF, Cheng TH. Inhibition of cyclic strain-induced endothelin-1 gene expression by resveratrol. *Hypertension* 2003; **42**: 1198-1205 [PMID: 14623829]
69 **Zou JG**, Wang ZR, Huang YZ, Cao KJ, Wu JM. Effect of red wine and wine polyphenol resveratrol on endothelial function in hypercholesterolemic rabbits. *Int J Mol Med* 2003; **11**: 317-320 [PMID: 12579333]
70 **Liu Z**, Song Y, Zhang X, Liu Z, Zhang W, Mao W, Wang W, Cui W, Zhang X, Jia X, Li N, Han C, Liu C. Effects of trans-resveratrol on hypertension-induced cardiac hypertrophy using the partially nephrectomized rat model. *Clin Exp Pharmacol Physiol* 2005; **32**: 1049-1054 [PMID: 16445570 DOI: 10.1111/j.1440-1681.2005.04303.x]
71 **Yang J**, Wang N, Li J, Zhang J, Feng P. Effects of resveratrol on NO secretion stimulated by insulin and its dependence on SIRT1 in high glucose cultured endothelial cells. *Endocrine* 2010; **37**: 365-372 [PMID: 20960276 DOI: 10.1007/s12020-010-9314-8]
72 **Fondevila C**, Busuttil RW, Kupiec-Weglinski JW. Hepatic ischemia/reperfusion injury--a fresh look. *Exp Mol Pathol* 2003; **74**: 86-93 [PMID: 12710939]
73 **Stein S**, Matter CM. Protective roles of SIRT1 in atherosclerosis. *Cell Cycle* 2011; **10**: 640-647 [PMID: 21293192 DOI: 10.4161/cc.10.4.14863]
74 **Stein S**, Schäfer N, Breitenstein A, Besler C, Winnik S, Lohmann C, Heinrich K, Brokopp CE, Handschin C, Landmesser U, Tanner FC, Lüscher TF, Matter CM. SIRT1 reduces endothelial activation without affecting vascular function in ApoE-/- mice. *Aging* (Albany NY) 2010; **2**: 353-360 [PMID: 20606253]
75 **Hernández-Jiménez M**, Hurtado O, Cuartero MI, Ballesteros I, Moraga A, Pradillo JM, McBurney MW, Lizasoain I, Moro MA. Silent information regulator 1 protects the brain against cerebral ischemic damage. *Stroke* 2013; **44**: 2333-2337 [PMID: 23723308 DOI: 10.1161/STROKEAHA.113.001715]
76 **Kosieradzki M**, Rowiński W. Ischemia/reperfusion injury in kidney transplantation: mechanisms and prevention. *Transplant Proc* 2008; **40**: 3279-3288 [PMID: 19100373 DOI: 10.1016/j.transproceed.2008.10.004]
77 **Bordone L**, Cohen D, Robinson A, Motta MC, van Veen E, Czopik A, Steele AD, Crowe H, Marmor S, Luo J, Gu W, Guarente L. SIRT1 transgenic mice show phenotypes resembling calorie restriction. *Aging Cell* 2007; **6**: 759-767 [PMID: 17877786 DOI: 10.1111/j.1474-9726.2007.00335.x]
78 **Satoh A**, Brace CS, Rensing N, Cliften P, Wozniak DF, Herzog ED, Yamada KA, Imai S. Sirt1 extends life span and delays aging in mice through the regulation of Nk2 homeobox 1 in the DMH and LH. *Cell Metab* 2013; **18**: 416-430 [PMID: 24011076 DOI: 10.1016/j.cmet.2013.07.013]
79 **Radak Z**, Koltai E, Taylor AW, Higuchi M, Kumagai S, Ohno H, Goto S, Boldogh I. Redox-regulating sirtuins in aging, caloric restriction, and exercise. *Free Radic Biol Med* 2013; **58**: 87-97 [PMID: 23339850 DOI: 10.1016/j.freeradbiomed.2013.01.004]
80 **Vaziri H**, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, Guarente L, Weinberg RA. hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* 2001; **107**: 149-159 [PMID: 11672523]
81 **Cheng HL**, Mostoslavsky R, Saito S, Manis JP, Gu Y, Patel P, Bronson R, Appella E, Alt FW, Chua KF. Developmental defects and p53 hyperacetylation in Sir2 homolog (SIRT1)-deficient mice. *Proc Natl Acad Sci USA* 2003; **100**: 10794-10799 [PMID: 12960381 DOI: 10.1073/pnas.1934713100]
82 **Anekonda TS**, Adamus G. Resveratrol prevents antibody-induced apoptotic death of retinal cells through upregulation of Sirt1 and Ku70. *BMC Res Notes* 2008; **1**: 122 [PMID: 19046449 DOI: 10.1186/1756-0500-1-122]
83 **Jeong J**, Juhn K, Lee H, Kim SH, Min BH, Lee KM, Cho MH, Park GH, Lee KH. SIRT1 promotes DNA repair activity and deacetylation of Ku70. *Exp Mol Med* 2007; **39**: 8-13 [PMID: 17334224]
84 **Fan H**, Yang HC, You L, Wang YY, He WJ, Hao CM. The histone deacetylase, SIRT1, contributes to the resistance of young mice to ischemia/reperfusion-induced acute kidney injury. *Kidney Int* 2013; **83**: 404-413 [PMID: 23302720 DOI: 10.1038/ki.2012.394]
85 **Brunet A**, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y,

Tran H, Ross SE, Mostoslavsky R, Cohen HY, Hu LS, Cheng HL, Jedrychowski MP, Gygi SP, Sinclair DA, Alt FW, Greenberg ME. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* 2004; **303**: 2011-2015 [PMID: 14976264 DOI: 10.1126/science.1094637]

86 **Yeung F**, Hoberg JE, Ramsey CS, Keller MD, Jones DR, Frye RA, Mayo MW. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. *EMBO J* 2004; **23**: 2369-2380 [PMID: 15152190 DOI: 10.1038/sj.emboj.7600244]

87 **Zhao G**, Ma H, Shen X, Xu GF, Zhu YL, Chen B, Tie R, Qu P, Lv Y, Zhang H, Yu J. Role of glycogen synthase kinase 3β in protective effect of propofol against hepatic ischemia-reperfusion injury. *J Surg Res* 2013; **185**: 388-398 [PMID: 23743186 DOI: 10.1016/j.jss.2013.05.004]

88 **Cui J**, Li Z, Qian LB, Gao Q, Wang J, Xue M, Lou XE, Bruce IC, Xia Q, Wang HP. Reducing the oxidative stress mediates the cardioprotection of bicyclol against ischemia-reperfusion injury in rats. *J Zhejiang Univ Sci B* 2013; **14**: 487-495 [PMID: 23733425 DOI: 10.1631/jzus.B1200263]

89 **Chen CJ**, Fu YC, Yu W, Wang W. SIRT3 protects cardiomyocytes from oxidative stress-mediated cell death by activating NF-κB. *Biochem Biophys Res Commun* 2013; **430**: 798-803 [PMID: 23201401 DOI: 10.1016/j.bbrc.2012.11.066]

90 **Pellegrini L**, Pucci B, Villanova L, Marino ML, Marfe G, Sansone L, Vernucci E, Bellizzi D, Reali V, Fini M, Russo MA, Tafani M. SIRT3 protects from hypoxia and staurosporine-mediated cell death by maintaining mitochondrial membrane potential and intracellular pH. *Cell Death Differ* 2012; **19**: 1815-1825 [PMID: 22595756 DOI: 10.1038/cdd.2012.62]

91 **Cheng Y**, Ren X, Gowda AS, Shan Y, Zhang L, Yuan YS, Patel R, Wu H, Huber-Keener K, Yang JW, Liu D, Spratt TE, Yang JM. Interaction of Sirt3 with OGG1 contributes to repair of mitochondrial DNA and protects from apoptotic cell death under oxidative stress. *Cell Death Dis* 2013; **4**: e731 [PMID: 23868064 DOI: 10.1038/cddis.2013.254]

92 **Yang H**, Yang T, Baur JA, Perez E, Matsui T, Carmona JJ, Lamming DW, Souza-Pinto NC, Bohr VA, Rosenzweig A, de Cabo R, Sauve AA, Sinclair DA. Nutrient-sensitive mitochondrial NAD+ levels dictate cell survival. *Cell* 2007; **130**: 1095-1107 [PMID: 17889652 DOI: 10.1016/j.cell.2007.07.035]

93 **Kim HS**, Patel K, Muldoon-Jacobs K, Bisht KS, Aykin-Burns N, Pennington JD, van der Meer R, Nguyen P, Savage J, Owens KM, Vassilopoulos A, Ozden O, Park SH, Singh KK, Abdulkadir SA, Spitz DR, Deng CX, Gius D. SIRT3 is a mitochondria-localized tumor suppressor required for maintenance of mitochondrial integrity and metabolism during stress. *Cancer Cell* 2010; **17**: 41-52 [PMID: 20129246 DOI: 10.1016/j.ccr.2009.11.023]

94 **Allison SJ**, Milner J. SIRT3 is pro-apoptotic and participates in distinct basal apoptotic pathways. *Cell Cycle* 2007; **6**: 2669-2677 [PMID: 17957139 DOI: 10.4161/cc.6.21.4866]

95 **Shi T**, Fan GQ, Xiao SD. SIRT3 reduces lipid accumulation via AMPK activation in human hepatic cells. *J Dig Dis* 2010; **11**: 55-62 [PMID: 20132432 DOI: 10.1111/j.1751-2980.2009.00416.x]

96 **Purushotham A**, Schug TT, Xu Q, Surapureddi S, Guo X, Li X. Hepatocyte-specific deletion of SIRT1 alters fatty acid metabolism and results in hepatic steatosis and inflammation. *Cell Metab* 2009; **9**: 327-338 [PMID: 19356714 DOI: 10.1016/j.cmet.2009.02.006]

97 **Raval AP**, Dave KR, Pérez-Pinzón MA. Resveratrol mimics ischemic preconditioning in the brain. *J Cereb Blood Flow Metab* 2006; **26**: 1141-1147 [PMID: 16395277 DOI: 10.1038/sj.jcbfm.9600262]

98 **Nadtochiy SM**, Redman E, Rahman I, Brookes PS. Lysine deacetylation in ischaemic preconditioning: the role of SIRT1. *Cardiovasc Res* 2011; **89**: 643-649 [PMID: 20823277 DOI: 10.1093/cvr/cvq287]

99 **Rane S**, He M, Sayed D, Vashistha H, Malhotra A, Sadoshima J, Vatner DE, Vatner SF, Abdellatif M. Downregulation of miR-199a derepresses hypoxia-inducible factor-1alpha and Sirtuin 1 and recapitulates hypoxia preconditioning in cardiac myocytes. *Circ Res* 2009; **104**: 879-886 [PMID: 19265035 DOI: 10.1161/CIRCRESAHA.108.193102]

100 **Yan W**, Fang Z, Yang Q, Dong H, Lu Y, Lei C, Xiong L. SirT1 mediates hyperbaric oxygen preconditioning-induced ischemic tolerance in rat brain. *J Cereb Blood Flow Metab* 2013; **33**: 396-406 [PMID: 23299244 DOI: 10.1038/jcbfm.2012.179]

101 **Zaouali MA**, Ben Mosbah I, Boncompagni E, Ben Abdennebi H, Mitjavila MT, Bartrons R, Freitas I, Rimola A, Roselló-Catafau J. Hypoxia inducible factor-1alpha accumulation in steatotic liver preservation: role of nitric oxide. *World J Gastroenterol* 2010; **16**: 3499-3509 [PMID: 20653058]

P- Reviewers: Du C, Zhang Y **S- Editor:** Ma YJ
L- Editor: Wang TQ **E- Editor:** Wang CH

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v19.i43.7603

World J Gastroenterol 2013 November 21; 19(43): 7603-7619
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Rare cystic liver lesions: A diagnostic and managing challenge

Andreas Bakoyiannis, Spiros Delis, Charina Triantopoulou, Christos Dervenis

Andreas Bakoyiannis, Spiros Delis, Christos Dervenis, First Surgical Department-HPB Surgical Unit, Konstantopouleion Agia Olga General Hospital, 14233 Athens, Greece
Charina Triantopoulou, Radiology Department, Konstantopouleion Agia Olga General Hospital, 14233 Athens, Greece
Author contributions: All authors contributed equally to this work; Bakoyiannis A and Delis S designed the research; Bakoyiannis A performed the research and wrote the paper; Triantopoulou C contributed regarding diagnostic work up and imaging modalities; and Dervenis C analyzed the data and reviewed the paper before submission.
Correspondence to: Andreas Bakoyiannis, Surgeon MD, Fist Surgical Department-HPB Surgical Unit, Konstantopouleion Agia Olga General Hospital, 3-5 Agia Olga street, 14233 Athens, Greece. andreasabaco@yahoo.gr
Telephone: +30-693-2668312 Fax: +30-210-5061372
Received: May 23, 2013 Revised: September 10, 2013
Accepted: September 16, 2013
Published online: November 21, 2013

Abstract

Cystic formations within the liver are a frequent finding among populations. Besides the common cystic lesions, like simple liver cysts, rare cystic liver lesions like cystadenocarcinoma should also be considered in the differential diagnosis. Thorough knowledge of each entity's nature and course are key elements to successful treatment. Detailed search in PubMed, Cochrane Database, and international published literature regarding rare cystic liver lesions was carried out. In our research are included not only primary rare lesions like cystadenoma, hydatid cyst, and polycystic liver disease, but also secondary ones like metastasis from gastrointestinal stromal tumors lesions. Up-to date knowledge regarding diagnosis and management of rare cystic liver lesions is provided. A diagnostic and therapeutic algorithm is also proposed. The need for a multidisciplinary approach by a team including radiologists and surgeons familiar with liver cystic entities, diagnostic tools, and treatment modalities is stressed. Patients with cystic liver lesions must be carefully evaluated by a multidisciplinary team, in order to receive the most appropriate treatment, since many cystic liver lesions have a malignant potential and evolution.

Key words: Liver cyst; Cystic tumor; Hepatic lesion; Gastrointestinal stromal tumors; Metastases; Cystadenoma; Cystadenocarcinoma; Hydatid cyst; Polycystic liver disease; Caroli; Echinococcus

Core tip: This paper reviews diagnosis differential diagnosis and management of rare cystic liver lesions which should be considered when a cystic hepatic lesion is identified. A diagnostic and therapeutic algorithm is provided. Patients with cystic liver lesions must be carefully evaluated by a multidisciplinary team, in order to receive the most appropriate treatment, since many cystic liver lesions have a malignant potential and evolution.

Bakoyiannis A, Delis S, Triantopoulou C, Dervenis C. Rare cystic liver lesions: A diagnostic and managing challenge. *World J Gastroenterol* 2013; 19(43): 7603-7619 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7603.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7603

INTRODUCTION

Cystic lesions within the liver have been reported to occur in up to 5% of the population[1]. Most of them are common and benign, but the possibility of a rarer cystic liver lesion, such as hepatobiliary cystadenoma (HC) or hepatobiliary cystadenocarcinoma (HCa), should not be overlooked. They can present with general or specific symptoms depending of the nature of the lesion, or they

can be silent and discovered accidentally[2]. In fact, most are found incidentally on imaging studies and tend to have a benign course, but a minority may cause symptoms, and rarely may be associated with serious morbidity and mortality[2]. The aim of our review is to focus upon the diagnostic and therapeutic algorithm of rare cystic lesions, including cystadenomas/cystadenocarcinomas, hydatid disease, polycystic liver disease, and metastatic neoplasms from the view of surgeons specialized in hepatobiliary surgery.

CYSTADENOMA AND CYSTADENOCARCINOMA

It is estimated that cystic neoplasms constitute approximately 5% of liver cysts, among which the malignancy is about 5%[2,3]. The overall incidence among hepatic malignant tumors is lower than 0.41%[2,3]. About 200 cases of HC, and a little more than half as many HCa, have been reported in the literature[4].

More than 85% of HC are reported in women, and typically in middle-aged persons in the fifth decade of life. HC is an unusual cystic lesion accounting for less than 5% of all biliary neoplasms[2,4]. The incidence of HCa is approximately 1 per 10 million patients. Malignant transformation is known to occur from HC to HCa. Older patients in the sixth decade of life are more likely to present with malignant tumors[2,4].

The histogenesis of HC is unknown, although a congenital origin is generally favored. A reactive process to some focal injury is still debated[5,6]. Pathologically, HC are multiloculated cysts with a stratified or pseudostratified non-ciliated columnar or cuboidal epithelium that contains mucous-producing cells. Papillary infolding is frequently present, and the mesenchyma underlying the tumor is usually hyper cellular, often with ovarian-appearing cells (85%-90%)[7-9]. The pre-malignant progression of HC is based on the histologic presence of intestinal metaplasia (IM), characterized by the presence of numerous goblet cells[10,11]. HC can easily be distinguished histologically from HCa, where a loss of epithelial nuclear stratification and a tubulo-papillary architecture with nuclear pleomorphism and atypia predominates. The malignant epithelium is multilayered with numerous papillary projections, and the confirmation of an invasion of the stroma confirms the diagnosis of HCa.

Regardless of the various diagnostic modalities, such a lesion (HC) may be difficult to distinguish preoperatively from an HCa[12].

The majority of HC is asymptomatic and discovered incidentally during radiographic studies, or they can present with symptoms related to tumor compression of adjacent organs due to their large size[2]. Patients presenting with symptoms generally complain of abdominal pain, abdominal distension, or a palpable mass. Less common presentations include intra-cystic hemorrhage, rupture, and fever from secondary bacterial infection. Any patient presenting with recurrence of liver cysts after treatment should be suspected of having a neoplastic cyst until proven otherwise[12].

HC and HCa should be differentiated from benign cystic hepatic lesions, including simple hepatic cyst, hepatic abscess, and echinococcal (hydatid) cyst. Simple hepatic cysts usually lack septa. Though hepatic abscesses and echinococcal cysts may appear similar to cystadenocarcinoma on diagnostic imaging, both infectious diseases are easily diagnosed through clinical and laboratory findings. Improvements in imaging techniques have helped to identify HC and HCa.

Ultrasound is an excellent modality that may delineate a simple cyst from other cystic lesions. Additionally, needle aspirates can be performed under ultrasound guidance. Simple cysts appear as anechoic unilocular fluid-filled space with imperceptible walls and posterior acoustic enhancement. A simple cyst is defined as a well-demarcated water attenuation lesion that does not enhance after the administration of intravenous contrast[2,4].

Contrast enhanced ultrasound (CEUS) is useful in assessing the vascularity of a mural nodule and making a distinction between a mural or septal nodule and intracystic debris[13]. In conventional ultrasound cystic lesions with solid components (septa, wall, mural nodule), this represents a wide range of rare entities like HC and HCa, as well as more common entities like simple liver cysts (after bleeding or with cell detritus), liver abscesses, or necrotic liver tumors[13]. CEUS can be informative regarding the vascularity of solid parts of a cystic lesion. Simple cysts, which are unclear in conventional ultrasound, might be identified in CEUS[13]. A cystic liver lesion without vascularization is most probably benign. CEUS is helpful in evaluating nodule vascularity and facilitates the final diagnosis[13].

On conventional ultrasound, a HC typically appears hypoechoic, with thickened irregular walls and occasional internal echoes. Xu *et al*[13], Lin *et al*[14], and Anderson *et al*[15] describe it as a well-defined unilocular, or more typically multilocular, cystic mass with mural or septal nodules in rare cases. On CEUS, a HC presents with septa enhancement during the arterial phase and hypoenhancement during the portal and late phases[13,14]. Cystadenocarcinoma, on the other hand, appears as a multilocular cystic mass with mural or septal nodules with thick and coarse calcifications on the septa on conventional ultrasound, while appearing on CEUS with septa enhancement during the arterial phase, mural or septal nodules enhancement and hypo-enhancement during the portal or late phase[13,14]. Xu *et al*[13] reported that on CEUS there is no significant difference between cystadenoma and cystadenocarcinoma regarding enhancement pattern and extent. Simple cysts, unlike HC, are virtually never septated[2,4]. Ultrasonography (US) is a very useful initial investigation in these patients as it demonstrates cystic lesions with thin internal septations, debris, projections, or mural nodes, and it can in most cases accurately distinguish simple from neoplastic cysts (Figure 1A).

Differential diagnosis between HC and HCa is dif-

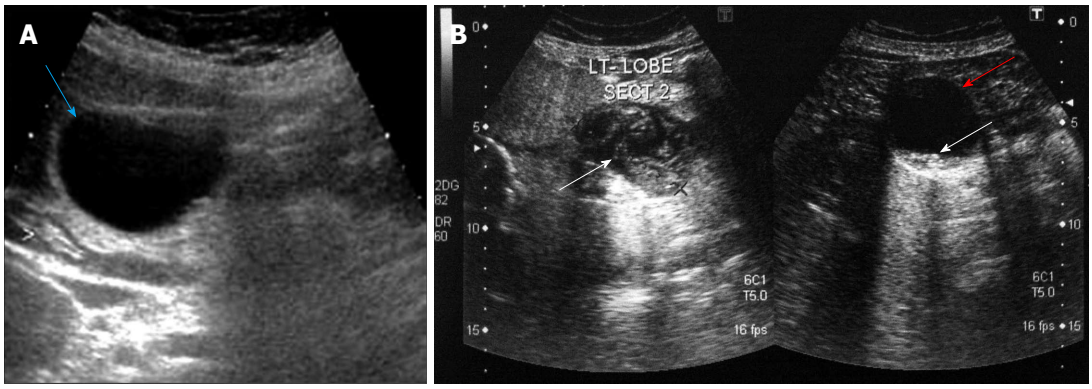


Figure 1 Ultrasound image. A: Showing an anechoic mass in the liver (light blue arrow), with a rather thin capsule (cystadenoma); B: Showing two echinococcal cysts. The first on the right (red arrow-right image) appears as an anechoic mass with hydatid sand (type CE1) (white arrow-right image), while in the second (on the left), the detached and folded endocyst membrane is obvious (type CE3) (white arrow-left image).

ficult. Although the presence of mural nodularity is not pathognomonic for cystadenocarcinoma, the absence of mural nodularity is suggestive of cystadenoma[15,16]. The diameter of the mural nodule (when it exists) in cystadenomas is much smaller (less than 1.0 cm) than mural or septal nodules in cystadenocarcinomas (larger than 1.0 cm)[13]. It seems that the presence of the internal septations and a mural or septal nodule, as well as the nodule diameter, might be diagnostic clues for differentiation between cystadenoma and cystadenocarcinoma[13]. The other differential-diagnostic characteristic between HC and HCa is that cystadenomas are more typically multilocular cystic lesions and cystadenocarcinomas more typically unilocular cystic or solid lesions[13].

Computed tomography (CT) is another useful modality to evaluate a cystic lesion of the liver. On a CT scan, a cystadenoma may be unilocular, multilocular, or may have septations. In a study from Vogt *et al*[3], all patients demonstrated septations within the cyst at the CT scan. The cyst wall is usually thickened or irregular, in contrast to a simple cyst. A cystadenoma may also have a smooth external surface and a thin wall (Figure 2A).

Magnetic resonance imaging (MRI) is very useful, as it demonstrates a well-defined lesion that does not enhance after the administration of intravenous gadolinium. On T1 images, the cyst shows a low signal; conversely on T2 weighted images, a very high intensity signal is observed. However, no specific information is gained towards pseudo-ovarian stroma detection[17].

Despite the various diagnostic modalities, it remains difficult to distinguish HCs from HCa on preoperative imaging; however, a significant solid component on the cystic wall suggests invasive malignant disease. Furthermore, HC can evolve into HCa after long periods lasting more than 10 years[18,19].

Liver enzymes and bilirubin are usually normal unless the biliary tree is compressed. The elevation of alkaline phosphate and bilirubin occurs in cases of bile duct obstruction. Carbohydrate antigen 19-9 may be elevated, but CEA and α-fetoprotein are usually normal[3,20]. It has been reported that most patients with cystadenocarcinoma have normal serum concentrations of CEA and CA 19-9. Moreover, the serum concentrations of these tumor markers can be elevated in patients with HC as well. Therefore, these serum tumor markers cannot distinguish HCa from HC.

Some authors have reported that fine needle aspiration cytology of the cyst contents is a good method for diagnosing cystic lesions[5,16]. In many studies, however, no malignant cells were recovered in patients with HCa who underwent intraoperative cytology examination. Thus, this procedure rarely generates a definitive diagnosis and carries the risk of pleural or peritoneal dissemination, and should therefore be avoided, especially when surgery is planned. The fluid of the cystic cavity often consists of a high-molecular-weight glycoprotein called mucin. However, hemorrhagic, bilious, clear, and mixed fluid contents have also been observed[5,16]. Aspiration and cyst fluid analysis for CEA and Ca 19-9 has been proved more useful than serum analysis[5,16]. Cyst fluid demonstrates marked, but variable, elevation in Ca 19-9 and moderate elevation of CEA[5,16]. Elevation of these cyst fluid tumor markers has high specificity and sensitivity in distinguishing HC from simple and echinococcal cysts.

HC has been treated by marsupialization, internal Roux-en-Y drainage, repeated needle aspirations, fenestration, or partial resection. All these methods have been associated with high rates of recurrence and complications including sepsis, continued growth, and progress to malignancy. Although the rate of malignant transformation is relatively low (5%-10%), all suspected HCs must be excised[16,21]. Liver resection with clear margins is strongly indicated due to the possibility of synchronous appearance of HCa at the borders of the cyst[16,21]. Enucleation is also acceptable. Reports supporting resection cite the low associated mortality of the procedure and the permanent relief of symptoms[16,21].

The majority of HC can be completely and safely excised by enucleation, including those that are centrally located. Once the cyst has been decompressed and the proper plane identified, enucleation can proceed without

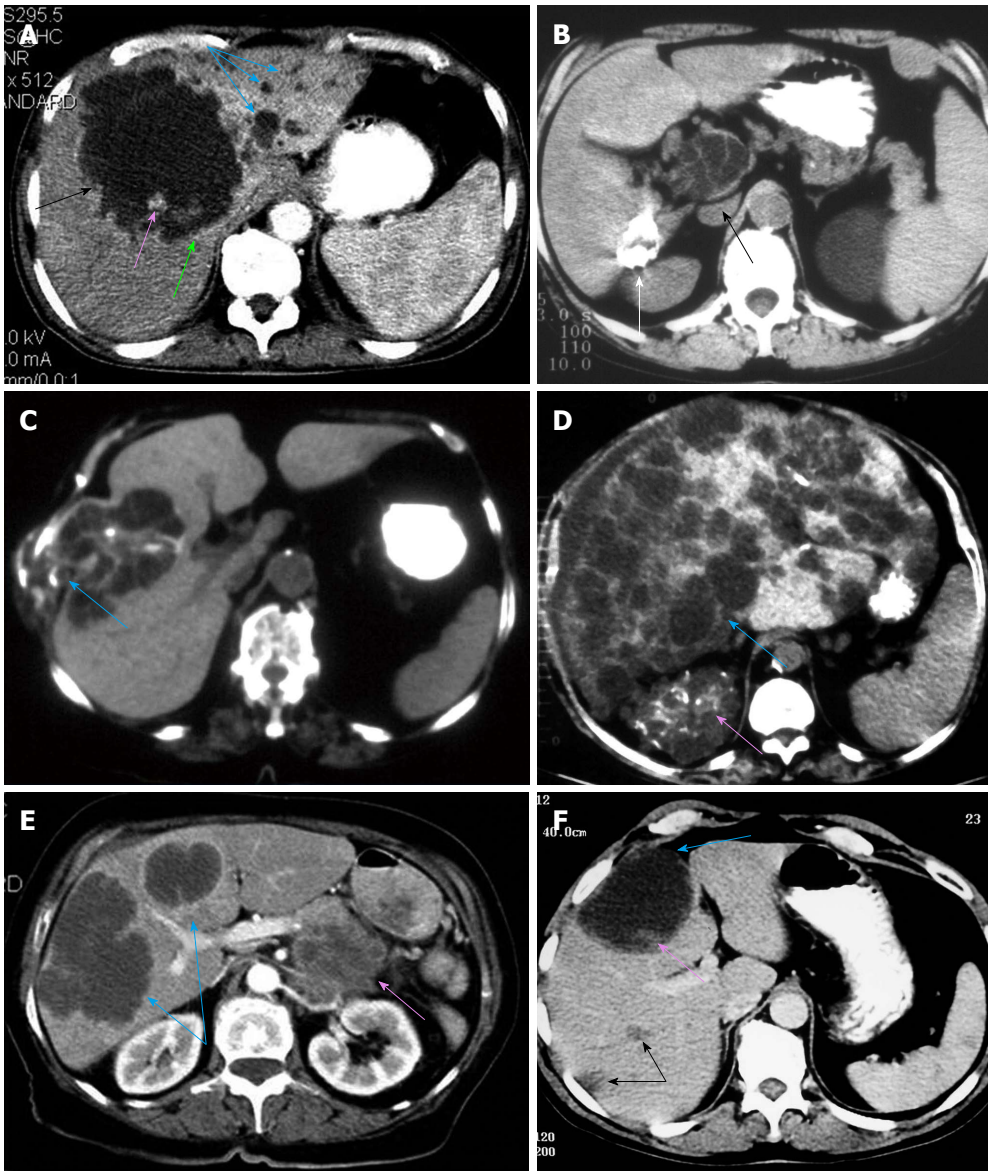


Figure 2 Computed tomography image. A: In liver segment Ⅳ there is a large cystic lesion (black arrow) causing compression with dilatation of biliary ducts (light blue arrows) in the left liver lobe. Peripheral contrast enhancement (light green arrow) as well as a nodule (pink arrow) is evident in this case of cystadenoma; B: Echinococcal disease evaluated. Two lesions are evident. The first in liver segment Ⅰ appears as a multilocular cystic lesion (black arrow) and the second in liver segment Ⅵ as a calcified mass with irregular margins (white arrow); C: Demonstrates direct infiltration of a liver hydatid cyst in the adjacent peritoneal surface and abdominal wall (light blue arrow); D: A typical case of multicystic disease with liver (light blue arrow) and kidney (pink arrow) involvement were very well depicted; E: Showing two large cystic-appearing liver lesions (light blue arrows) in a case of a metastatic pancreatic cystadenocarcinoma that is also evident (pink arrow); F: A large cystic lesion (light blue arrow) with a small solid component at the periphery (pink arrow), as well as two small hypodense liver lesions (black arrows), are seen on this image, in a case of proven gastrointestinal stromal tumors metastatic lesions.

significant blood loss. If the possibility of hemorrhage is high due to adjacent major venous vascular structures, enucleation can be completed with either inflow occlusion (Pringle maneuver) or total vascular exclusion. In the era of laparoscopic surgery, a laparoscopic frozen section biopsy of the cyst wall is feasible. If the frozen section is consistent with a simple, benign cyst, laparoscopic partial excision is adequate. If the biopsy demonstrates HC, then complete excision is necessary. However, frozen section biopsies are not always accurate due to inconsistency and discontinuity of the pathological epithelium[3,7,22]. Frozen sections cannot definitely exclude or confirm the diagnosis of HC, especially in the case of HCa[3,7].

The only potentially curative treatment for HCa is complete removal, usually by a major liver resection with 1-cm margins. Reported survival rates for HCa range from 25% to 100% (87% disease free) at 5 years[4]. It has been reported that patients with HCa who have invasion of the liver parenchyma or neighboring organs have a poor prognosis[4]. Asahara *et al* have reported that the prognosis of patients with HCa is poor when the tumor lacks mesenchymal stroma[2,4]. Absence of mesenchymal stroma in HCa appears to be associated with aggressive disease behavior (*i.e.*, rapid dissemination or distant metastasis)[2,4].

HYDATID DISEASE

Human cystic echinococcosis, or hydatid cyst disease, is a zoonosis caused by the larval cestode *Echinococcus granulosus, Echinococcus multilocularis*, or *Echinococcus vogeli*. *E. granulosus* produces unilocular cystic lesions, whereas *E. multilocularis* and *E. vogeli* produce multilocular alveolar cysts[23,24]. Dogs are the definitive hosts for *E. Granulosus*, with sheep being the major intermediate host (yaks, goats, and camels are other relevant intermediate hosts). Man is only incidentally infected when ingesting tapeworm eggs[24]. The eggs penetrate the intestinal wall, with the resulting larvae infiltrating the blood and lymphatic circulation system. Then, through the portal vein into the liver, lungs, and other tissues, the larvae develop into hydatid cysts[25,26].

The liver is the most frequent site for the cystic lesions (52%-77%) seen in hydatid disease, followed by the lung (10%-40%), brain, and other viscera[24,26,27]. The disease may remain silent for many years before coming into medical attention as an incidental imaging finding, or it may present with complications.

The diagnosis of uncomplicated hepatic hydatid disease is based on clinical suspicion, with special attention paid to factors such as the patient's residence, place of origin and occupation in order to identify high-risk patients. The symptoms depend on the size, location, and development stage of the cyst[26,28]. Pain in the right upper quadrant or the epigastrium is the most common symptom, whereas hepatomegaly and a palpable mass are the most common signs. Nonspecific symptoms such as fatigue, fever, nausea, or dyspepsia may also be present. Patients with complicated hepatic hydatid disease may present with fever, jaundice, or anaphylactic symptoms, depending on the complication[26,29].

Acute cholangitis is the most common syndrome when the hydatid cysts rupture in the biliary tract. Rupture of a cyst may produce fever, pruritus, eosinophilia, or fatal anaphylaxis[23]. Lower chest pain, a productive cough, and hemoptysis are the most frequent symptoms when there is thoracic involvement. Bilioptysis is diagnostic of a biliobroncheal fistula[25].

General blood tests are not specific except in complicated disease, whereas a high white blood cell count with eosinophilia are possible findings. Hepatic parameters are normal except in the case of biliary compression[30]. Serologic tests such as hemagglutination, latex agglutination, and enzyme-linked immunosorbent assay (ELISA), are associated with a high incidence of false-negative and false-positive results[28]. Nevertheless, the detection of specific antigens and immune complexes of the cyst with ELISA yields a positive result in more than 90% of patients. Specific IgE antibodies are demonstrated with ELISA and the radioallergosorbent test is positive in the presence of active disease. Confirmatory tests such as arc-5 immunoelectrophoresis and immunoblotting use parasite-specific antigens. The positivity rate with arc-5 immunoelectrophoresis is as high as 91.1%[26,29]. The Casoni and Weinberg tests are no longer used for the diagnostic workup, mainly due to their low sensitivity[29].

The indirect immunofluorescence assay (IFA) first reported by Coudert *et al* is specific and sensitive, especially in cases of hepatic cystic hydatidosis. This easy-to-do assay can be achieved in less than 2.5 h and is the most sensitive test in more than 95% of patients with hepatic cystic hydatidosis[30]. The diagnosis of hydatidosis by molecular biology is based on the polymerase chain reaction and the technique needs to be evaluated. Based on the choice of primers and probes, molecular biology can differentiate *E. granulosus* from *E. multilocularis* in clinical samples[30].

False-positive results have been described in some patients with tumors, for which there is no explanation as yet, whereas false-negative results are observed when cysts are calcified, even if fertile and corresponding to the lack of antigenic stimulation. Serologic tests do not supplant clinical or imaging investigations but they can, however, confirm the hydatic origin of a cyst. Specific antibodies increase 4-6 wk after surgery, after which they decrease slowly for the next 12-18 mo. The decrease in specific antibodies is too irregular to be a good witness of recovery or relapse, however. Persistently high specific antibody titers or a secondary increase in the antibody titers 6 to 12 mo after surgery indicate a relapse[30].

Standard chest and abdomen radiographs may reveal an elevated diaphragm and concentric calcifications in the cyst wall. Liver scanning was an important diagnostic tool during the 1970s. Since then, US and CT have replaced scanning and are considered the first choice in the diagnostic armamentarium. These methods are helpful for determining complications as well[29]. MRI and endoscopic retrograde cholangiopancreatography (ERCP) can prove helpful during the diagnostic approach.

US, a noninvasive, readily available, sensitive, and cost-effective imaging technique, should be the diagnostic method of choice. US is helpful for defining the internal structure, number, and location of the cysts and the presence of complications (Figure 1B). The specificity of US is in the range of 90%. Several authors have proposed an ultrasonographic classification of hepatic hydatid disease (Table 1)[26,31]. Classification was standardized by the World Health Organization-Informal Working Group on Echinococcosis (WHO-IWGE) in 2001 (Table 2)[26]. According to the five categories noted in the classification of Gharbi types: Ⅱ and Ⅲ are characteristic of hydatid cysts, types Ⅰ and Ⅴ are suggestive of hydatid cysts in endemic areas, and type Ⅳ simulates a pseudotumor[25].

CT is a helpful tool for confirming the diagnosis, essentially when an ultrasound examination shows a type Ⅳ sonographic pattern[25]. It provides information equivalent to that derived by US, but it shows the location and depth of the cyst within the liver more accurately (Figure 2B and C). Moreover it can reveal calcified cystic walls[28], daughter cysts, and exogenous cysts, as well as evaluate their volume and density. CT is essential for planning surgical treatment, especially when a minimally invasive approach is to be used[26,29]. Imaging findings on CT depend on the stage of cyst growth and the *Echinococcus* species

Table 1 Gharbi classification of cystic hydatid disease

Type	Ultrasonographic features and patterns
I	Pure fluid collection
II	Fluid collection with a split wall (water-lily sign)
III	Fluid collection with septa (honeycomb sign)
IV	Heterogeneous echographic patterns
V	Reflecting thick walls

Table 2 World Health Organization-Informal Working Group on Echinococcosis

Type	Ultrasonographic features and patterns
CL	Unilocular cystic lesion with uniform anechoic content, cyst wall not visible
CE1	Unilocular cystic lesion with uniform anechoic content, cyst wall visible, snowflake sign
CE2	Multivesicular, multiseptated cysts, daughter cysts present, honeycomb sign
CE3	Unilocular cyst containing liquid with a floating membrane inside, daughter cysts may be present, water-lily sign
CE4	Cysts with heterogeneous hypoechoic or hyperechoic degenerative contents, no daughter cysts
CE5	Cysts characterized by a thick calcified wall, which is arch-shaped, producing a cone-shaped shadow; degree of calcification varies from partial to complete

involved. Hepatic involvement by *E. multilocularis* is characterized by a different appearance than *E. granulosus*, consisting of an infiltrating solid mass composed of multiple cysts and indistinct margins. Infection by *E. granulosus* usually forms a single cyst, with or without daughter cysts[23].

Although MRI can be helpful for demonstrating the lesion in the liver (Figure 3), it does not provide additional information in hepatic lesions and is not cost-effective when compared with either US or CT[26,29]. However, both CT and MRI have high specificity and sensitivity in the detection and differential diagnosis of hepatic cysts and extracapsular (satellite) cysts[28].

The ideal treatment for hepatic hydatid disease should completely eliminate the parasite and prevent recurrence of the disease with minimum morbidity and mortality. There are three available therapeutic modalities for hepatic hydatid cysts; systemic chemotherapy, surgery, and the treatment known as "puncture, aspiration, injection, reaspiration" (PAIR). Chemotherapy and PAIR are recommended as alternatives to surgery, especially for patients who cannot tolerate or refuse surgery. However, surgery is still the first choice of treatment for hepatic hydatid cysts. Selection of the most appropriate treatment depends on the patient's health status, the nature of the cyst(s) (considering number, size, location, and presence of complications), and the available resources and expertise[26].

Mebendazole (MBZ) was the first benzimidazole carbamate agent found to have *in vivo* activity in hydatid disease. The drug interferes with mechanisms of glucose absorption through the wall of the parasite, leading to

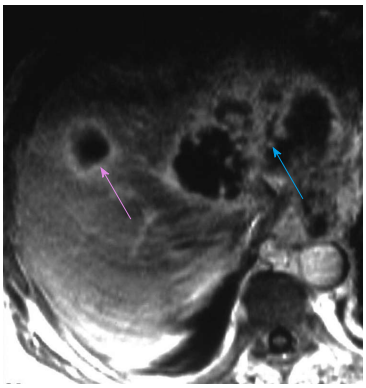


Figure 3 Magnetic resonance T1-w image shows an echinococcal cyst as a multiloculated cystic liver lesion, indicative of the presence of daughter cysts (light blue arrow). A second smaller unilocular lesion with peripheral contrast enhancement is also seen (pink arrow).

glycogen depletion and subsequent degenerative changes in the mitochondria and endoplasmic reticulum of the germinal cells[32]. Albendazole (ABZ) is more active *in vitro* than MBZ and has improved gastrointestinal absorption and bioavailability, as well as reports of better clinical results[32]. Although orally administered, ABZ results in high serum concentrations and penetration into cyst contents is erratic. Currently, ABZ chemotherapy as the primary treatment may be considered for patients who are not acceptable candidates for surgery, have inoperable, recurrent, peritoneal or multiple liver cysts within the whole liver, have multiple cysts in several organs, refuse surgery or percutaneous drainage, and perhaps, for asymptomatic individuals[32].

Both drugs may decrease the size of hydatid cysts and lead to the sterilization of cyst contents in some cases; however, without concomitant drainage, clinical and radiographic resolution is unpredictable and occurs in less than half of treated patients[24]. Hepatic and hematologic toxicities are the most frequent serious adverse effects of ABZ and MBZ. Treatment of hepatic cystic echinococcosis with MBZ or ABZ alone is not as effective as a combined chemotherapy-drainage approach[24,33]. Clinical and radiographic improvement (in most studies defined as a > 25% reduction in cyst size, membrane separation, or cyst calcification) is seen frequently, but complete cure (*i.e.*, cyst disappearance) generally occurs in less than half of patients treated with anti-parasitic monotherapy[24,33].

According to the WHO guidelines, chemotherapy is indicated for inoperable primary liver or lung echinococcosis, for patients with multiple cysts in two or more organs, and for peritoneal cysts. Another important indication for chemotherapy is the prevention of secondary echinococcosis. Preoperative use of ABZ or MBZ can reduce the risk of recurrence of cystic echinococcosis and facilitate the operation. Concomitant chemotherapy is also recommended for PAIR. Chemotherapy is contraindicated for large cysts that are at risk of rupture (superficially situated, infected cysts) and for inactive or calcified cysts[34].

The usually recommended oral dosage of ABZ is

10-15 mg/kg per day in two divided doses for several 1-mo courses separated by 14-d intervals. The usual oral dosage of MBZ is given as 500 mg tablets in daily doses of 40-50 mg/kg (in three divided doses) for at least 3-6 mo. Better intestinal absorption of benzimidazole compounds is gained by administering it with a fat-rich meal or by combining it with cimetidine. Medical and laboratory examinations for adverse reactions are initially necessary every 2 wk and then monthly[35].

A third antiparasitic agent, praziquantel, has had limited use in the treatment of hydatid cysts of the liver. The drug increases the permeability of the parasite's cell membrane to calcium, resulting in strong contractions and paralysis of the musculature leading to detachment from host tissue. In humans, it has favorable pharmacokinetics when given in a dose of 50 mg/kg either once weekly or every two weeks. There are few clinical studies documenting the efficacy of praziquantel in humans, however several of these have suggested that the use of praziquantel in combination with MBZ or ABZ is more effective and perhaps, more rapid than with benzimidazole alone (47.4% *vs* 36.4%) after only 2-6 mo of drug therapy[24].

Surgery was defined as the only definitive and curative modality by the WHO-IWGE in 1996[33]. The goals of surgery in hydatid disease are to inactivate the cestode parasites, evacuate the cyst cavity, remove the germinal layer, and obliterate the residual cavity. Surgical interventions consist of open conservative, radical, and laparoscopic approaches[24]. Conservative techniques involve drainage, marsupialization, capitonnage, deroofing, partial simple cystectomy, and open or closed total cystectomy with or without omentoplasty[24]. The conservative procedures are safer and easier to perform[25]. Radical procedures include total pericystectomy, partial hepatectomy, or lobectomy[24]. Although it seems logical that radical operations would be associated with higher intra- and postoperative morbidity but less frequent recurrence, recent studies have shown that radical surgery is not associated with a high complication rate[26].

Laparoscopic drainage of hepatic hydatid cysts is a "minimally invasive" surgical technique that appears safe and effective. It has the theoretic advantages of a shorter hospital stay, lower incidence of wound infection, and less postoperative pain, but the disadvantages of difficult accessibility to the various locations, increased risk of cyst content spillage, and the difficulty of aspirating the cyst content of the thick, degenerated cyst contents, especially in some WHO-IWGE CE3 and CE4 cysts. Thus, choosing the best candidates for the laparoscopic approach requires careful evaluation of the cystic disease[25,26]. Whichever technique is used, a benzimidazole agent is best administered before any surgery in an attempt to sterilize the cyst contents and reduce the risk of anaphylaxis and dissemination[24].

Meticulous packing of the operative field is necessary irrespective of the surgical technique employed, as is the use of solutions that kill the infective scoleces and protoscolices of the parasite residing within the hydatid cyst, or potentially leaking from the cyst during surgical manipulation. Various scolicidal solutions used in surgical (and percutaneous) approaches include: hypertonic saline (3%-20%), povidone-iodine, hydrogen peroxide, iodine, formalin, silver nitrate, ethyl alcohol, and ABZ. These scolicides can be used alone or in combination[24].

Potential major complications associated with the surgical treatment of hepatic hydatid cysts include postoperative hemorrhage, bile exudation from the residual cyst cavity, incisional fistula formation, cholangitis, wound infection, sepsis, incisional fistulae, pulmonary complications such as pneumonia and pulmonary embolization, complications of anesthesia, and death[24].

ERCP is used as a diagnostic and therapeutic tool in the management of biliary tract-complicated hepatic hydatid cysts. Preoperatively, ERCP defines biliary tract-related complications and allows the assessment and management of acute conditions, including acute cholangitis and biliary obstruction, so that elective surgery can be performed later. When combined with sphincterotomy, this drains the cyst cavity and helps prevent postoperative biliary fistula. Postoperatively, ERCP allows visualization of distorted anatomy in recurrent cases, helps clarify the etiology of ongoing or recurrent biliopancreatic symptoms and biochemical abnormalities, allows endoscopic management of a biliary fistula, and enables treatment of secondary biliary strictures by stenting[26].

The treatment modality that we prefer using in our department with optimal results is the evacuation of the cavity with careful removal of the laminated membrane and the daughter cysts in order to avoid spillage. The cyst cavity is obliterated by omentoplasty or capitonnage, and the site is drained externally by suction catheter. Partial cystectomy and internal drainage with a Roux-en-Y intracystic hepaticojejunostomy is performed when large ducts had been disrupted due to large cysts. Preoperative ERCP is performed when communication between the cyst cavity and biliary tree is suspected, and endoscopic sphincterotomy is performed in cases of obstruction.

The minimally-invasive technique of puncture, aspiration of cyst, injection of hypertonic saline and/or absolute alcohol, and re-aspiration (PAIR), described initially by Voros *et al*[28] and Falagas *et al*[32], is an alternative to major interventional procedures. PAIR treatment satisfies all the goals of surgery in hydatid disease, but substitutes germinal membrane sclerosing and separation using scolicides for surgical removal. PAIR drainage is best performed under continuous ultrasonographic or CT guidance.

Patients undergoing PAIR typically receive oral ABZ that is administered 24-4 h before intervention and 15-30 d after intervention according to cyst size[34]. Different scolicidal solutions can be used in PAIR, although hypertonic saline is most commonly employed. Hypertonic saline (in 5%-30% concentrations) exerts its scolicidal effect by creating a strong osmotic gradient across the outer cuticular membrane of the protoscolex, which

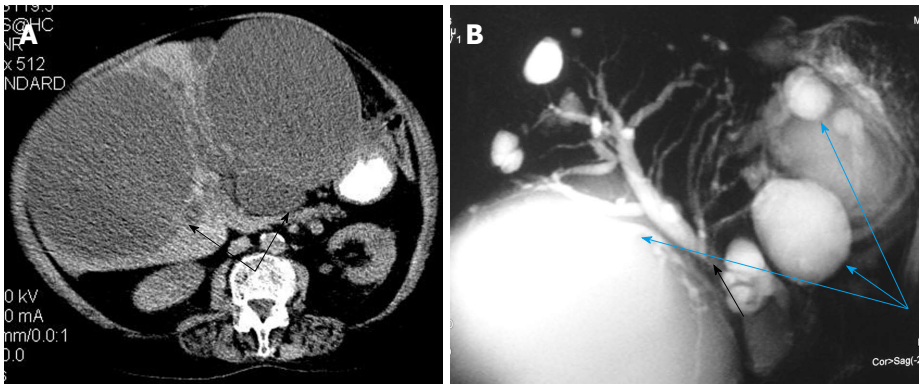


Figure 4 Image. A: Computed tomography image in a case of multicystic disease, showing two large dominant cysts causing mild intrahepatic biliary dilatation (black arrows); B: Magnetic resonance cholangiopancreatography image shows multiple hepatic cysts (light blue arrows) while the common bile duct seems compressed between the two larger cysts (black arrow).

causes its lysis. For multiseptate Type Ⅲ cysts or large cysts over 6 cm in size, some authors advocate the use of absolute alcohol because it is a more effective sclerosing agent than hypertonic saline, may destroy daughter cysts not killed by saline, and may also result in a more rapid involution of the cyst cavity. Alcohol should not be used, however, if pre-existing biliary communication is suspected or documented, as the agent may cause a chemical cholangitis[33].

With PAIR, cyst fluid or operative tissue specimens are immediately subjected to cytologic, histopathologic, and parasitologic examinations after aspiration or catheter drainage in order to confirm the diagnosis and assess the success of the drainage procedure.

Complications after PAIR therapy, such as infections, are generally well tolerated and can be managed with systemic antimicrobial therapy. Leakage during drainage may lead to fever, urticaria, transient hypotension, or anaphylaxis, but these can be anticipated and effectively managed with antipyretics, Ⅳ fluids, antihistamines, and subcutaneous epinephrine. Cyst-biliary communications (biliary rupture and fistula formation) developing after PAIR and caused by cyst decompression, can usually be managed endoscopically[24]. For patients who underwent PAIR as a primary procedure, a total complication rate of 14.7% and a recurrence rate of 1.57% have been reported[28].

In conclusion, compared to patients undergoing surgical intervention for cystic hepatic echinococcosis, PAIR plus ABZ is associated with greater clinical and parasitologic efficacy, less major and minor morbidity whenever it is indicated (*i.e.*, for non-echoic lesion ⩾ 5 cm in diameter (CE1), cysts with daughter cysts (CE2), and/or with detachment of membranes (CE3). Surgery may be reserved for patients with hydatid cysts refractory to PAIR because of secondary bacterial infection or difficult-to-manage cyst-biliary communication or obstruction[24,34].

POLYCYSTIC LIVER DISEASE

Polycystic liver disease (PLD) is inherited as an autosomal dominant trait presenting in adulthood and is more common in women[36]. Autosomal dominant polycystic disease is genetically heterogeneous, with mutations in two distinct genes predisposing to the combination of renal and liver cysts (AD-PKD1 and AD-PKD2)[36,37]. PLD is genetically linked to protein kinase C substrate 80K-H and SEC63[38]. The cysts in PLD can also increase in size and number during pregnancy or simultaneously with the use of exogenous female steroid hormones[39].

Most patients are asymptomatic and do not require treatment. Some patients develop massive hepatic cystic disease and become clinically symptomatic, which is associated with increased liver volume and adjacent visceral compression. Usually patients suffer from chronic dull abdominal pain, satiety, weight loss, dyspnea, physical disability, and descensus[36,40]. Liver function tests are usually normal except for mild elevation in ALP or γ-GT[36,40]. Liver failure or complications of advanced liver disease, such as infection or intracystic hemorrhage, are rare. Less than 5% of patients have acute medical complications. These consist of cyst hemorrhage, rupture, infection, uterine prolapse due to displacement, obstructive jaundice, portal hypertension, ascites, and Budd-Chiari syndrome[19,36,40-42]. Even with marked hepatosplenomegaly and portal hypertension, liver function is well preserved in PLD. Ascites may be present and usually results from hepatic venous flow obstruction. Diagnosis is confirmed with US and CT imaging (Figure 2D), which along with MRI provides the surgeon with valuable preoperative information, such as the location of infected or hemorrhagic cysts that may be responsible for symptoms[40] (Figure 4). Treatment should be considered in cases of persistent symptoms or associated complications.

Cyst aspiration with sclerosis, open or laparoscopic cyst fenestration, combined hepatic resection and fenestration, liver transplantation, and recent medical treatment with somatostatin analogues, are possible therapeutic options based on the type of PLD[19,36,40,42-46]. Aspiration, combined with ethanol instillation to induce sclerosis of the cyst lining epithelium, can be effective in patients with a few dominant cysts (Type I PLD - few large cysts

greater than 7 cm). Open or laparoscopic cyst fenestration with omentoplasty is another modality of treatment that can be performed in patients with more diffuse PLD (Type Ⅱ-multiple medium cysts 5-7 cm in diameter). Patients with small cysts throughout the liver have a greater risk of persistence and/or recurrence of symptoms[19,42]. Postoperative morbidity consists of temporary ascites, pleural effusion, and rarer biliary leakage[40].

Combined hepatic resection and fenestration is more effective for reducing the hepatic mass and relieving gastric compression. This procedure has an advantage in the case of massive hepatomegaly with associated gastric compression[40,47,48]. Resection addresses the problem of liver mass, but poses significant risk of bile duct injury, vascular compromise, and liver insufficiency, as cysts markedly distort intrahepatic anatomy. In particular, ascites has been troublesome due to continued cyst secretion from residual fenestrated cysts, disruption of intrahepatic lymphatics, and partial venous outflow obstruction. Candidates for combined resection/fenestration should have at least two adjacent liver segments not affected by cysts and have normal liver function. Furthermore, these patients should be managed by experienced hepatobiliary surgeons at institutions with advanced intensive care, as well as interventional radiological and gastroenterology support.

Liver transplantation has been performed in rare cases, especially when the above-mentioned interventions are not an option. In patients who harbor diffuse PLD, orthotopic liver transplantation (OLT) is effective, but inherently assumes the risks of long-term immunosuppression and rejection. OLT is indicated for patients with progressive PLD after resection/fenestration, patients with concurrent liver dysfunction and renal failure, and patients with diffuse PLD without segmental sparing. Although symptomatic relief from hepatomegaly occurred in all surviving patients, long term follow up addressing quality of life, hepatorenal function, immunosuppressive complications, and survival is limited[42,49].

Regarding the results of invasive methods, in case series it is noted that aspiration and sclerosis of individual liver cysts reduced liver volume by 19% in patients with 1 or more large dominant liver cysts[50]. Reduction of liver volume is reported to be as high as 12.5% when laparoscopic fenestration is used, but the complication rate reported is also high (0%-33%)[51-55].

The drawbacks of invasive procedures in treating PLD are their partial effectiveness, their related morbidity and mortality and, most importantly, the fact that they do not change the natural course of the disease as symptoms recur due to growth of new cysts or re-growth of treated ones[41].

Several studies have reported the positive effects of somatostatin analogues in decreasing liver and kidney growth in PKD and ADPLD over a treatment period of minimum 6 mo[43-46].

Somatostatin may reduce cyst development through several mechanisms[45]: (1) by inhibiting secretin release from the pancreas[56]; (2) by inhibiting secretin-induced cAMP generation and fluid secretion in cholangiocytes[57-59]; (3) by vasopressin-induced cAMP generation and water permeability in collecting ducts[60-63] by its effects on Gi protein-coupled receptors; and (4) by suppressing the expression of IGF-1, vascular endothelial growth factor, and other cystogenic growth factors and downstreaming signaling from their receptors[60-64].

Ruggenenti *et al*[43] in 2005 showed that kidney volume increased by 2.2%-3.7% during active treatment with octreotide LAR compared with 5.9%-5.4% ($P < 0.01$) while on placebo. Octreotide LAR (40 mg intramuscularly every 4 wk) was given for 6 mo in 12 adult polycystic kidney disease (ADPKD) patients with advanced renal disease (mean total kidney volume 2435 mL, mean serum creatinine 1.9 mg/dL)[43].

In 2009, van Keimpema *et al*[44], tested lanreotide for treating PLD (120 mg subcutaneously every 4 wk) for 6 mo in 54 patients with PLD (32 ADPKD and 22 ADPLD). He concluded that liver volume decreased by 2.9% (from 4606 to 4471 mL) in the lanreotide group, whereas it increased by 1.6% (from 4689 to 4895 mL) in the placebo group ($P < 0.01$)[44]. In the 32 patients with ADPKD, total kidney volume decreased by 1.5% (from 1000 to 983 mL) in the lanreotide group, whereas it increased by 3.4% (from 1115 to 1165 mL) in the placebo group ($P < 0.02$)[44].

The results of the clinical trial reported in 2010 by Hogan *et al*[45] showed that administration of octreotide LAR for 1 year induced a moderate but significant reduction in liver volume, inhibited the growth of polycystic kidneys, and improved quality of life in patients with ADPKD and/or ADPLD, with low toxicity and few side effects.

In 2012, Hogan *et al*[46] reported their results in treating patients with PLD with Octreotide LAR for 2 years. He concluded that his study further substantiates the positive effects of somatostatin analogs in reducing TLV (6.08% overall reduction in TLV in the first year), demonstrating their safety and efficacious over a 2-year period in individuals with ADPKD or ADPLD, many of whom had chronic renal insufficiency[46]. While OctLAR inhibited renal enlargement within the first year of treatment, it appeared to lose effectiveness during Year 2. While the results of OctLAR therapy on TLV were clearly positive and results on TKV may show some benefit, he did not detect any positive effect of OctLAR on GFR[46].

The administration of octreotide or lanreotide has been generally well tolerated in all studies, with mostly mild, predictable, and dose-dependent gastrointestinal side effects. Patients undergoing long-term octreotide treatment should be monitored for cholelithiasis symptoms or signs; a known complication[45,65]. Alopecia, symptomatic bradycardia, and steatorrhea are other known adverse events associated with somatostatin analogue treatment[66-73].

CYSTIC METASTASES

Many cystic tumors may metastasize to the liver (*e.g.*, pancreatic or ovary cystadenocarcinomas) (Figure 2E). Other liver metastatic lesions which can appear cystic usually originate from rapidly growing hypervascular tumors (sarcoma, melanoma, and neuroendocrine tumors) and appear so due to necrosis and cystic degeneration[74]. Despite the fact that these metastatic sites often show cystic characteristics, in most cases the differential diagnosis between them and benign liver cysts is easily made with the contribution of computed tomography.

Of particular interest are the liver metastases from gastrointestinal stromal tumors (GIST) that may appear cystic even before treatment (Figure 2F).

GIST is the most common mesenchymal tumor of the gastrointestinal tract, accounting for 1%-3% of all gastrointestinal malignancies[75-77]. GIST can arise anywhere along the GI tract, but is most common in the stomach (50%-70%) and small bowel (25%-35%)[78].

Despite its less aggressive pathologic nature, GIST can metastasize after a long remission period. When GIST originates in the small bowel it behaves in a more aggressive manner. The most common site for metastases is the liver and the peritoneal cavity[76,77,79], but it can also occur in bones, lungs, skin, and lymph nodes. Data from the MD Anderson Cancer Center report 18% of patients presented with metastatic disease[80]. Liver metastases are commonly multiple, distributed in both lobes, and more frequently detected synchronously with the primary tumor than metachronously. Case reports showed further metachronous liver metastases being detected after more than 10 years (13 years after gastrectomy for gastric GIST, 17 years after resection for retroperitoneum GIST, and 12 years after surgery for rectal GIST)[77].

GIST mostly affects males between the ages 40 and 70 and are usually found incidentally. Features at clinical presentation depend on tumor size. Large or advanced lesions may present with a variety of clinical findings, including bleeding, abdominal pain, early satiety, bowel obstruction, or perforation. Bowel obstruction is reported in up to 30% of clinical series, but accounts for less than 10% of presentations in most reports[81].

The initial workup should include history and physical examination, appropriate imaging (*i.e.*, chest, abdominal and pelvic CT with contrast and/or MRI), endoscopy in selected cases of primary gastric mass, endoscopic ultrasound, liver function tests, full blood counts, and surgical assessment of tumor resectability. On CT scans, metastases within the liver developed lower attenuation than that of the normal surrounding parenchyma. Liver metastases are hypervascular in 92% of patients, and rapidly become cystic following therapy with imatinib.

GIST and GIST liver metastases are soft and fragile, and biopsy may cause tumor hemorrhage or dissemination. The decision to obtain a biopsy should be based on data regarding the stage of the disease and the clinician' s suspicion of other malignancies. If the diagnosis is in doubt, neo-adjuvant therapy using IM is put under consideration, but if there is synchronous metastatic disease, then biopsy is essential[77].

Before 2001, the only effective therapy was surgery alone. The development of clinically effective inhibitors targeting the trans-membrane receptor tyrosine kinase KIT radically changed the management of advanced (locally advanced and metastatic) disease. IM, a selective inhibitor of tyrosine kinase, has revolutionized the management of this disease in recent years. Laboratory studies revealed significant molecular heterogeneity among GIST[82-84]. In 2010, a meta-analysis showed that most patients with different genotypes of GIST and KIT exon 11-mutants benefit from the individualized treatment of imatinib[85].

Imatinib has become the first line treatment for recurrent and/or metastatic disease[79]. Another meta-analysis comparing the efficacy of imatinib given either once (400 mg) or twice daily, revealed that the higher dose offers a progression-free survival advantage among patients with exon 9 mutations[86], but that the overall survival was the same in the two groups of patients.

Nowadays imatinib therapy and surgical intervention are combined to give patients better disease free and survival rates. Surgical intervention in patients responding to medical therapy may provide a complete cure[87,88]. Complete pathological response to imatinib alone occurs in less than 5% of patients[87,88]. Surgery has the best results when offered to patients with lesions responsive to 6 mo imatinib therapy. CT with or without PET can be used to assess the therapeutic effect. MSKCC patients with lesions responsive to imatinib in one study had a 2-year progression free survival of 61% and a 2-year overall survival of 100% after surgical resection. In contrast, the 2-year survival was 36% in patients resistant to imatinib therapy[89]. Raut *et al*[90] also reported that debulking surgery may prolong survival in patients who are either responsive to imatinib or have limited radiographic progression, but it has poor or no result in patients with progressive metastatic disease. Gronchi *et al*[91] reported in 2007 that surgery may be of value to patients who develop responsive or stable disease while on preoperative imatinib therapy.

Regarding the timing of surgical resection, Suzuki *et al*[92] reported a complete resection rate of 31.4% after IM therapy for a period of 6.9-37.5 mo (mean 10 mo). They also emphasized that surgical resection for IM- responsive recurrent or metastatic disease should be considered as early as possible before the development of progression and secondary resistance to IM[92]. Surgical resection 6-12 mo after the start of IM treatment is recommended among responders[79]. However a large tumor may prohibit resection because of the risk of postoperative liver failure. An option to counteract this phenomenon is the use of portal vein embolization (PVE). In general, the median time for detection of further metastases following resection of liver metastases, is 12 mo after the initial hepatectomy[77,79]. Therefore, careful evaluation of the liver is critical during the first year post-hepatectomy.

Radio-frequency ablation, microwave ablation, and

hepatic artery embolization are other treatment modalities that can normally be used in patients with unresectable disease or in those who cannot undergo surgical excision due to co-morbidities.

CYSTIC HEPATOCELLULAR CARCINOMA

Rarely hepatocellular carcinoma (HCC) can have a cystic appearance, due to necrosis and cystic degeneration in cases of rapidly growing tumors. Co-existing liver cirrhosis and specific HCC imaging characteristics, such as hypervascularity of solid components and tumor invasion of the portal and hepatic veins, can help to reach the correct diagnosis[74]. Typically the conventional ultrasound would reveal a heterogeneous echogenic lesion with a hypoechoic rim and peripheral or internal arterial flow signals in a liver cirrhosis background. The CEUS would reveal a heterogenous hyper-enhancement during the arterial phase and hypo-enhancement during the portal and late phases[14].

CAROLI DISEASE

Caroli disease (CD) is a benign congenital disorder, characterized by unilobular or bilobular segmental cystic dilatation of the intrahepatic biliary tract. The first report was by Todd in 1818, but Jacques Caroli in 1958 defined the disease precisely with its different types[93]. The estimated incidence of Caroli disease is 1 in 1000000, with males and females being equally affected and more than 80% of patients presenting before 30 years of age[94].

There are two forms of the disease, one associated with congenital hepatic fibrosis, also called Caroli syndrome, and the other a simple form occurring alone. Recent studies suggest that the simple form may be as common as congenital hepatic fibrosis[95,96]. It is characterized by segmental cystic dilatation of the intrahepatic ducts, increased incidence of biliary lithiasis, cholangitis, and liver abscesses. Absence of cirrhosis and portal hypertension is typical[94]. Various renal disorders have been described in association with this liver disease, including autosomal polycystic kidney disease, medullary sponge kidney, and medullary cystic disease[93,95].

Mode of inheritance is still unclear, but in the majority of cases it is transmitted in autosomal recessive fashion[93]. Caroli disease is associated with an increased risk of cholangiocarcinoma, with the reported incidence of malignancy ranging from 5% to 10%. The estimated risk is 100 times greater than that of the general population and is triggered by long-standing inflammation and chronic injury of the biliary epithelium[94,97]. Although present from birth, the disease usually remains asymptomatic during the first 20 years, and may also remain so throughout life[94]. However when symptomatic, a significant number of these patients present significant loss in their quality of life and clinical course.

The disease is frequently noted by recurrent fever, jaundice, and/or pain in the right hypochondrium[97]. A literature review found recurrent acute cholangitis as the main mode of presentation in 64% of patients[94] and the most life-threatening complication of CD. Usually caused by gram-negative bacilli, it has a recurrent course and, despite different antibiotic associations, medical treatment is often not satisfactory[93]. Patients with Caroli syndrome, on the other hand, usually present early in life, with complications of portal hypertension, mainly variceal bleeding, hypersplenism, or portal hypertension in 20%-50% of cases[97].

Laboratory studies typically show an elevation of serum alkaline phosphatase, direct bilirubin, and a leukocytosis with a predominance of neutrophils. Hepatic synthetic function is well-preserved initially, but may be affected by progressive liver damage due to recurrent cholangitis and biliary obstruction. Coagulopathy from vitamin K malabsorption may occur in cholestatic patients[94].

Histologically, the main macroscopic and microscopic features of CD are: non-obstructive, localized dilatation of the bile ducts; intraluminal bulbar protrusions of the ductal wall and intra ductal vascular tracts containing patent portal venous; and hepatic arterial channels that traverse the true lumen and terminate within the lumen[94]. The diagnosis of CD therefore rests on demonstrating that the cystic lesions are in continuity with the biliary tree. This can be done by imaging studies such as isotope scan, MRCP, CT scan, US, ERCP, and PTC.

The classical finding of CD is finding that a cold area on 99mTC sulfur colloid scan becomes hot on 99mTc DISIDA scan[94]. MRCP presents advantages in depicting the entire biliary tree. Three main patterns of CD are identified in MRCP: (1) multiple cystic ectasias connected with fusiform dilatations; (2) isolated fusiform dilatations with multiple calculi; and (3) solitary dilatation of the left bile ducts with cysts and multiple calculi[93] (Figure 5).

A CT scan shows central dot signs in CD patients. The fibrovascular bundles containing portal vein radical and a branch of hepatic artery bridging the saccule appear as central dots or a linear streak. This central dot sign described on a CT scan is suggested as a pathognomonic finding in CD, and can also be demonstrated on US[94]. ERCP is the method with the highest sensitivity in the diagnosis of CD. The cholangiographic features of Caroli disease are well established as saccular or fusiform dilatation of the intrahepatic bile ducts. Irregular bile duct walls, strictures, and stones may be present. Therefore, direct cholangiography is considered the method of choice for an accurate diagnosis of CD[94]. With PTC, the diagnosis can be made confidently when the large intrahepatic branches have focal or segmental involvement with cystic outpouchings in which the contrast medium collects. False-positive findings are rare when PTC is used[93].

The treatment of CD depends on the clinical features and location of the biliary abnormalities. It seems more than justified to advocate a rather aggressive surgical strategy in symptomatic patients who have had several

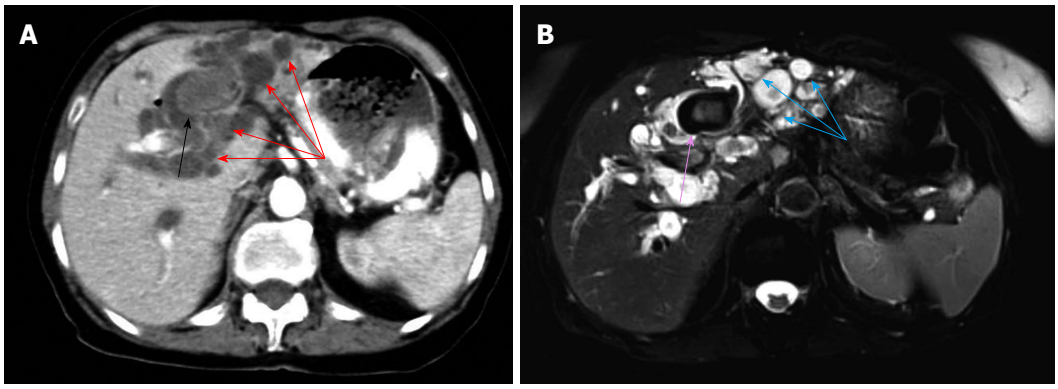


Figure 5 A case of Caroli disease. A: On computed tomography. A large intra-biliary stone (black arrow) is evident in the dilated ducts (red arrows); B: On magnetic resonance imaging. A large intra-biliary stone (pink arrow) is evident in the dilated ducts (blue arrows).

futile conservative treatment attempts[98]. The localized forms, which involve either the whole of the left or the right half of the liver, are curable by surgery. They should be treated by hemi-hepatectomy, left or right, with associated treatment of any problem affecting the common duct[94]. The procedure is associated with low morbidity and virtual no mortality. There is no report of malignant tumors arising after surgical resection[98].

Diffuse involvement of both lobes can be treated with conservative management in asymptomatic patients, with appropriate antibiotics for cholangitis and ursodeoxycholic acid therapy for litholysis in cases of intrahepatic cholelithiasis, endoscopic therapy (sphincterotomy for clearance of intrahepatic stones), and internal biliary bypass procedure[94]. These patients in whom there is no indication for liver resection or transplantation should at least be followed up regularly on an outpatient basis to detect any kind of deterioration or malignant transformation as early as possible[98].

Bilateral disease complicated by recurrent cholangitis, cirrhosis, or both, together with symptoms of associated hepatic fibrosis, do not find the same solution, and it is often difficult to manage. Emergency surgery in the presence of acute cholangitis and deteriorating liver function is associated with high mortality (20%-40%) and morbidity (44%-80%)[94].

It seems that liver transplantation or living donor transplantation is an effective therapeutic option and possibly the only and ultimate management option for these patients with end-stage diffuse Caroli disease providing gratifying long-term results[94,97,98]. OLT has become a therapeutic option which, aside from the better long-term outcome, can prevent the development of cholangiocarcinoma[96].

DISCUSSION

Liver cystic lesions consist of a heterogeneous group of disorders. Rare liver cystic lesions such as cystadenoma, hydatid cyst, polycystic liver disease, Caroli disease, and cystic liver metastases pose several dilemmas to the practicing surgeon or physician. It is very important that awareness and a high index of suspicion for rare diseases and HC are present, so as to provide as accurate a diagnosis as possible. Since our diagnostic tools have become more powerful and accurate, our adequate knowledge of the nature, evolution, confirmation, and treatment of all the possible pathological entities in the differential diagnosis becomes more necessary than ever.

The use of CEUS in diagnoses of liver lesions has shown promising results, providing more accurate images than conventional ultrasound[99-102]. The discrimination between malignant and benign lesions is easier and more accurate than in a conventional ultrasound.

Complete non-enhancement throughout three phases of CEUS or sustained enhancement in the portal and late phases is noticed in most benign lesions[14]. Conversely, hypoenhancement in the late phase is seen in malignancies[14]. Real time CEUS improves the capability of discrimination between benign and malignant complex cystic focal liver lesions[14]. It has been shown that CEUS can greatly improve the diagnostic accuracy of focal liver lesions compared with conventional ultrasound[14].

Contrast-enhanced ultrasound has also been used for diagnostics of the biliary system. In 2009, Xu[100] summarized the methodology, image interpretation, enhancement pattern, clinical usefulness, and indications for CEUS in the biliary system.

The first important step, regarding liver cystic lesions, is to make a definitive diagnosis of the nature of the cystic lesion. The second is determining whether the patient's symptoms are related to the cystic lesion. The third is deciding whether and when to initiate therapy for the lesion. Finally, a number of treatment options are available, leading to the fourth issue, which is deciding the appropriate therapy for the patient.

Ideally the cystic liver lesions should be handled by a multidisciplinary team familiar with liver diseases, consisting of interventional radiologist, interventional gastroenterologist, surgeon, clinical oncologist, and pathologist. In our opinion this is the way that even rare entities can be identified and treated promptly. The algorithm used in our department for managing such cystic lesions is provided in Figure 6.

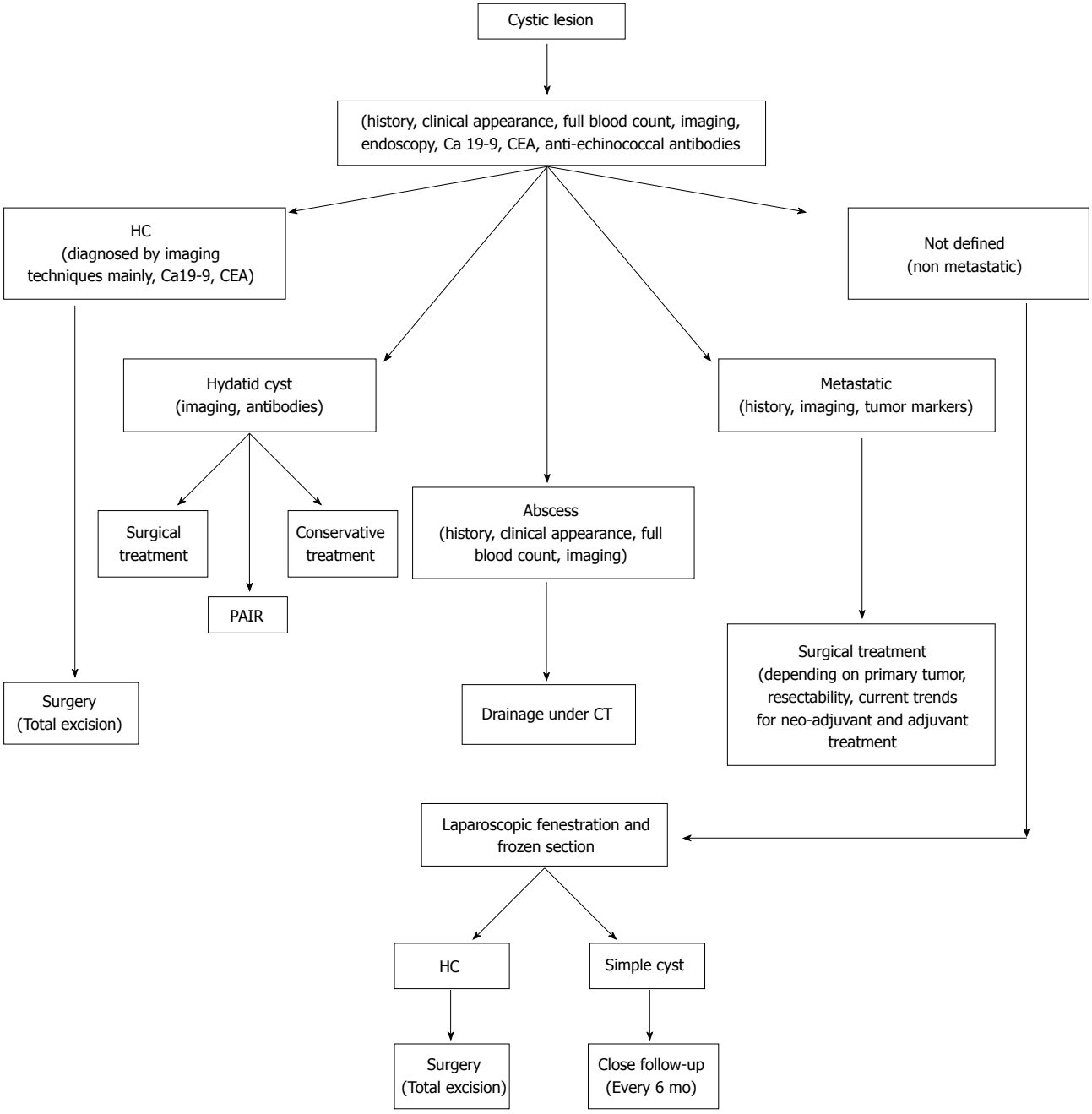


Figure 6 Liver cystic lesions management algorithm.

CONCLUSION

Cystic liver lesions require accurate pre-treatment diagnosis in order to select the appropriate therapy for each patient, as they can represent benign or malignant formations. It is best that a specialized team deals with cystic liver lesions so that diagnosis and treatment are accurate and focused. Specifically, rare entities require accurate diagnosis and management, as they can pose a malignant impact.

REFERENCES

1 **Caremani M**, Vincenti A, Benci A, Sassoli S, Tacconi D. Echographic epidemiology of non-parasitic hepatic cysts. *J Clin Ultrasound* 1993; **21**: 115-118 [PMID: 8381130 DOI: 10.1002/jcu.1870210207]

2 **Hai S**, Hirohashi K, Uenishi T, Yamamoto T, Shuto T, Tanaka H, Kubo S, Tanaka S, Kinoshita H. Surgical management of cystic hepatic neoplasms. *J Gastroenterol* 2003; **38**: 759-764 [PMID: 14505130 DOI: 10.1007/s00535-003-1142-7]

3 **Vogt DP**, Henderson JM, Chmielewski E. Cystadenoma and cystadenocarcinoma of the liver: a single center experience. *J Am Coll Surg* 2005; **200**: 727-733 [PMID: 15848365 DOI: 10.1016/j.jamcollsurg.2005.01.005]

4 **Carson JG**, Huerta S, Butler JA. Hepatobiliary cystadenoma: a case report and a review of the literature. *Curr Surg* 2006; **63**: 285-289 [PMID: 16843782 DOI: 10.1016/j.cursur.2006.03.001]

5 **Ishak KG**, Willis GW, Cummins SD, Bullock AA. Biliary

cystadenoma and cystadenocarcinoma: report of 14 cases and review of the literature. *Cancer* 1977; **39**: 322-338 [PMID: 318915]
6 **Subramony C**, Herrera GA, Turbat-Herrera EA. Hepatobiliary cystadenoma. A study of five cases with reference to histogenesis. *Arch Pathol Lab Med* 1993; **117**: 1036-1042 [PMID: 8215827]
7 **Hansman MF**, Ryan JA, Holmes JH, Hogan S, Lee FT, Kramer D, Biehl T. Management and long-term follow-up of hepatic cysts. *Am J Surg* 2001; **181**: 404-410 [PMID: 11448430 DOI: 10.1016/S0002-9610(01)00611-0]
8 **Marcial MA**, Hauser SC, Cibas ES, Braver J. Intrahepatic biliary cystadenoma. Clinical, radiological, and pathological findings. *Dig Dis Sci* 1986; **31**: 884-888 [PMID: 3731980 DOI: 10.1007/BF01296059]
9 **Ferell L**. Benign and malignant tumors of the liver. In: Odze RD, Goldblum JR, Crawford JM. Surgical Pathology of the GI Tract, Liver, Biliary Tract, and Pancreas. Collingwood: Saunders; 2004: 1015-1016
10 **Koffron A**, Rao S, Ferrario M, Abecassis M. Intrahepatic biliary cystadenoma: role of cyst fluid analysis and surgical management in the laparoscopic era. *Surgery* 2004; **136**: 926-936 [PMID: 15467680 DOI: 10.1016/j.surg.2004.06.031]
11 **Thomas JA**, Scriven MW, Puntis MC, Jasani B, Williams GT. Elevated serum CA 19-9 levels in hepatobiliary cystadenoma with mesenchymal stroma. Two case reports with immunohistochemical confirmation. *Cancer* 1992; **70**: 1841-1846 [PMID: 1525758]
12 **Delis SG**, Touloumis Z, Bakoyiannis A, Tassopoulos N, Paraskeva K, Athanassiou K, Safioleas M, Dervenis C. Intrahepatic biliary cystadenoma: a need for radical resection. *Eur J Gastroenterol Hepatol* 2008; **20**: 10-14 [PMID: 18090983 DOI: 10.1097/MEG.0b013e3282f16a76]
13 **Xu HX**, Lu MD, Liu LN, Zhang YF, Guo LH, Liu C, Wang S. Imaging features of intrahepatic biliary cystadenoma and cystadenocarcinoma on B-mode and contrast-enhanced ultrasound. *Ultraschall Med* 2012; **33**: E241-E249 [PMID: 23154870 DOI: 10.1055/s-0031-1299276]
14 **Lin MX**, Xu HX, Lu MD, Xie XY, Chen LD, Xu ZF, Liu GJ, Xie XH, Liang JY, Wang Z. Diagnostic performance of contrast-enhanced ultrasound for complex cystic focal liver lesions: blinded reader study. *Eur Radiol* 2009; **19**: 358-369 [PMID: 18795298 DOI: 10.1007/s00330-008-1166-8]
15 **Anderson SW**, Kruskal JB, Kane RA. Benign hepatic tumors and iatrogenic pseudotumors. *Radiographics* 2009; **29**: 211-229 [PMID: 19168846 DOI: 10.1148/rg.291085099]
16 **Thomas KT**, Welch D, Trueblood A, Sulur P, Wise P, Gorden DL, Chari RS, Wright JK, Washington K, Pinson CW. Effective treatment of biliary cystadenoma. *Ann Surg* 2005; **241**: 769-73; discussion 773-5 [PMID: 15849512 DOI: 10.1097/01.sla.0000161982.57360.1b]
17 **Dixon E**, Sutherland FR, Mitchell P, McKinnon G, Nayak V. Cystadenomas of the liver: a spectrum of disease. *Can J Surg* 2001; **44**: 371-376 [PMID: 11603751]
18 **Korobkin M**, Stephens DH, Lee JK, Stanley RJ, Fishman EK, Francis IR, Alpern MB, Rynties M. Biliary cystadenoma and cystadenocarcinoma: CT and sonographic findings. *AJR Am J Roentgenol* 1989; **153**: 507-511 [PMID: 2669463 DOI: 10.2214/ajr.153.3.507]
19 **Mortelé KJ**, Ros PR. Cystic focal liver lesions in the adult: differential CT and MR imaging features. *Radiographics* 2001; **21**: 895-910 [PMID: 11452064]
20 **Zhang M**, Yu J, Yan S, Zheng SS. Cystadenocarcinoma of the liver: a case report. *Hepatobiliary Pancreat Dis Int* 2005; **4**: 464-467 [PMID: 16109538]
21 **Fiamingo P**, Veroux M, Cillo U, Basso S, Buffone A, D' Amico DF. Incidental cystadenoma after laparoscopic treatment of hepatic cysts: which strategy? *Surg Laparosc Endosc Percutan Tech* 2004; **14**: 282-284 [PMID: 15492659 DOI: 10.1097/00129689-200410000-00011]
22 **Veroux M**, Fiamingo P, Cillo U, Tedeschi U, Brolese A, Veroux P, Basso S, Buffone A, D'Amico DF. Cystadenoma and laparoscopic surgery for hepatic cystic disease: a need for laparotomy? *Surg Endosc* 2005; **19**: 1077-1081 [PMID: 16021374 DOI: 10.1007/s00464-004-2229-9]
23 **Czermak BV**, Akhan O, Hiemetzberger R, Zelger B, Vogel W, Jaschke W, Rieger M, Kim SY, Lim JH. Echinococcosis of the liver. *Abdom Imaging* 2008; **33**: 133-143 [PMID: 17912581 DOI: 10.1007/s00261-007-9331-0]
24 **Smego RA**, Sebanego P. Treatment options for hepatic cystic echinococcosis. *Int J Infect Dis* 2005; **9**: 69-76 [PMID: 15708321 DOI: 10.1016/j.ijid.2004.08.001]
25 **Dziri C**, Haouet K, Fingerhut A. Treatment of hydatid cyst of the liver: where is the evidence? *World J Surg* 2004; **28**: 731-736 [PMID: 15457348 DOI: 10.1007/s00268-004-7516-z]
26 **Sayek I**, Tirnaksiz MB, Dogan R. Cystic hydatid disease: current trends in diagnosis and management. *Surg Today* 2004; **34**: 987-996 [PMID: 15580379 DOI: 10.1007/s00595-004-2830-5]
27 **Delis SG**, Bakoyiannis A, Exintabelones T, Triantopoulou C, Papailiou J, Dervenis C. Rare localizations of the hydatid disease. Experience from a single center. *J Gastrointest Surg* 2007; **11**: 195-198 [PMID: 17390172 DOI: 10.1007/s11605-006-0036-4]
28 **Voros D**, Katsarelias D, Polymeneas G, Polydorou A, Pistiolis L, Kalovidouris A, Gouliamos A. Treatment of hydatid liver disease. *Surg Infect* (Larchmt) 2007; **8**: 621-627 [PMID: 18171123 DOI: 10.1089/sur.2006.0070]
29 **Sayek I**, Onat D. Diagnosis and treatment of uncomplicated hydatid cyst of the liver. *World J Surg* 2001; **25**: 21-27 [PMID: 11213152]
30 **Biava MF**, Dao A, Fortier B. Laboratory diagnosis of cystic hydatic disease. *World J Surg* 2001; **25**: 10-14 [PMID: 11213147]
31 **Gharbi HA**, Hassine W, Brauner MW, Dupuch K. Ultrasound examination of the hydatic liver. *Radiology* 1981; **139**: 459-463 [PMID: 7220891]
32 **Falagas ME**, Bliziotis IA. Albendazole for the treatment of human echinococcosis: a review of comparative clinical trials. *Am J Med Sci* 2007; **334**: 171-179 [PMID: 17873530 DOI: 10.1097/MAJ.0b013e31814252f8]
33 **Rozanes I**, Güven K, Acunaş B, Emre A. Cystic echinococcal liver disease: new insights into an old disease and an algorithm for therapy planning. *Cardiovasc Intervent Radiol* 2007; **30**: 1112-1116 [PMID: 17533534 DOI: 10.1007/s00270-007-9081-y]
34 Guidelines for treatment of cystic and alveolar echinococcosis in humans. WHO Informal Working Group on Echinococcosis. *Bull World Health Organ* 1996; **74**: 231-242 [PMID: 8789923]
35 **Saimot AG**. Medical treatment of liver hydatidosis. *World J Surg* 2001; **25**: 15-20 [PMID: 11213151]
36 **Everson GT**, Taylor MR, Doctor RB. Polycystic disease of the liver. *Hepatology* 2004; **40**: 774-782 [PMID: 15382167 DOI: 10.1002/hep.20431]
37 **Rosenfeld L**, Bonny C, Kallita M, Heng AE, Deteix P, Bommelaer G, Abergel A. [Polycystic liver disease and its main complications]. *Gastroenterol Clin Biol* 2002; **26**: 1097-1106 [PMID: 12520197]
38 **Janssen MJ**, Salomon J, Te Morsche RH, Drenth JP. Loss of heterozygosity is present in SEC63 germline carriers with polycystic liver disease. *PLoS One* 2012; **7**: e50324 [PMID: 23209713 DOI: 10.1371/journal.pone.0050324]
39 **Bistritz L**, Tamboli C, Bigam D, Bain VG. Polycystic liver disease: experience at a teaching hospital. *Am J Gastroenterol* 2005; **100**: 2212-2217 [PMID: 16181371 DOI: 10.1111/j.1572-0241.2005.50258.x]
40 **Morgan DE**, Lockhart ME, Canon CL, Holcombe MP, Bynon JS. Polycystic liver disease: multimodality imaging for complications and transplant evaluation. *Radiograph-*

ics 2006; **26**: 1655-168; quiz 1655 [PMID: 17102042 DOI: 10.1148/rg.266065013]
41 **Arnold HL**, Harrison SA. New advances in evaluation and management of patients with polycystic liver disease. *Am J Gastroenterol* 2005; **100**: 2569-2582 [PMID: 16279915 DOI: 10.1111/j.1572-0241.2005.00263.x]
42 **Dranssart M**, Cognet F, Mousson C, Cercueil JP, Rifle G, Krause D. MR cholangiography in the evaluation of hepatic and biliary abnormalities in autosomal dominant polycystic kidney disease: study of 93 patients. *J Comput Assist Tomogr* 2006; **26**: 237-242 [PMID: 11884780 DOI: 10.1097/00004728-200203000-00013]
43 **Ruggenenti P**, Remuzzi A, Ondei P, Fasolini G, Antiga L, Ene-Iordache B, Remuzzi G, Epstein FH. Safety and efficacy of long-acting somatostatin treatment in autosomal-dominant polycystic kidney disease. *Kidney Int* 2005; **68**: 206-216 [PMID: 15954910 DOI: 10.1111/j.1523-1755.2005.00395.x]
44 **van Keimpema L**, Nevens F, Vanslembrouck R, van Oijen MG, Hoffmann AL, Dekker HM, de Man RA, Drenth JP. Lanreotide reduces the volume of polycystic liver: a randomized, double-blind, placebo-controlled trial. *Gastroenterology* 2009; **137**: 1661-8.e1-2 [PMID: 19646443 DOI: 10.1053/j.gastro.2009.07.052]
45 **Hogan MC**, Masyuk TV, Page LJ, Kubly VJ, Bergstralh EJ, Li X, Kim B, King BF, Glockner J, Holmes DR, Rossetti S, Harris PC, LaRusso NF, Torres VE. Randomized clinical trial of long-acting somatostatin for autosomal dominant polycystic kidney and liver disease. *J Am Soc Nephrol* 2010; **21**: 1052-1061 [PMID: 20431041 DOI: 10.1681/ASN.2009121291]
46 **Hogan MC**, Masyuk TV, Page L, Holmes DR, Li X, Bergstralh EJ, Irazabal MV, Kim B, King BF, Glockner JF, Larusso NF, Torres VE. Somatostatin analog therapy for severe polycystic liver disease: results after 2 years. *Nephrol Dial Transplant* 2012; **27**: 3532-3539 [PMID: 22773240 DOI: 10.1093/ndt/gfs152]
47 **Iwatsuki S**, Starzl TE. Personal experience with 411 hepatic resections. *Ann Surg* 1988; **208**: 421-434 [PMID: 3178330 DOI: 10.1097/00000658-198810000-00004]
48 **Tikkakoski T**, Mäkelä JT, Leinonen S, Päivänsalo M, Merikanto J, Karttunen A, Siniluoto T, Kairaluoma MI. Treatment of symptomatic congenital hepatic cysts with single-session percutaneous drainage and ethanol sclerosis: technique and outcome. *J Vasc Interv Radiol* 1996; **7**: 235-239 [PMID: 9007803 DOI: 10.1016/S1051-0443(96)70767-4]
49 **Que F**, Nagorney DM, Gross JB, Torres VE. Liver resection and cyst fenestration in the treatment of severe polycystic liver disease. *Gastroenterology* 1995; **108**: 487-494 [PMID: 7835591 DOI: 10.1016/0016-5085(95)90078-0]
50 **van Keimpema L**, de Koning DB, Strijk SP, Drenth JP. Aspiration-sclerotherapy results in effective control of liver volume in patients with liver cysts. *Dig Dis Sci* 2008; **53**: 2251-2257 [PMID: 18299984 DOI: 10.1007/s10620-007-0121-x]
51 **van Keimpema L**, Ruurda JP, Ernst MF, van Geffen HJ, Drenth JP. Laparoscopic fenestration of liver cysts in polycystic liver disease results in a median volume reduction of 12.5%. *J Gastrointest Surg* 2008; **12**: 477-482 [PMID: 17957434 DOI: 10.1007/s11605-007-0376-8]
52 **Fiamingo P**, Tedeschi U, Veroux M, Cillo U, Brolese A, Da Rold A, Madia C, Zanus G, D'Amico DF. Laparoscopic treatment of simple hepatic cysts and polycystic liver disease. *Surg Endosc* 2003; **17**: 623-626 [PMID: 12574922]
53 **Gigot JF**, Jadoul P, Que F, Van Beers BE, Etienne J, Horsmans Y, Collard A, Geubel A, Pringot J, Kestens PJ. Adult polycystic liver disease: is fenestration the most adequate operation for long-term management? *Ann Surg* 1997; **225**: 286-294 [PMID: 9060585 DOI: 10.1097/00000658-199703000-00008]
54 **Konstadoulakis MM**, Gomatos IP, Albanopoulos K, Alexakis N, Leandros E. Laparoscopic fenestration for the treatment of patients with severe adult polycystic liver disease. *Am J Surg* 2005; **189**: 71-75 [PMID: 15701496 DOI: 10.1016/j.amjsurg.2004.03.011]
55 **Robinson TN**, Stiegmann GV, Everson GT. Laparoscopic palliation of polycystic liver disease. *Surg Endosc* 2005; **19**: 130-132 [PMID: 15531969 DOI: 10.1007/s00464-004-8813-1]
56 **Li JP**, Lee KY, Chang TM, Chey WY. MEK inhibits secretin release and pancreatic secretion: roles of secretin-releasing peptide and somatostatin. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G890-G896 [PMID: 11292597]
57 **Gong AY**, Tietz PS, Muff MA, Splinter PL, Huebert RC, Strowski MZ, Chen XM, LaRusso NF. Somatostatin stimulates ductal bile absorption and inhibits ductal bile secretion in mice via SSTR2 on cholangiocytes. *Am J Physiol Cell Physiol* 2003; **284**: C1205-C1214 [PMID: 12676656 DOI: 10.1152/ajpcell.00313.2002]
58 **Tietz PS**, Holman RT, Miller LJ, LaRusso NF. Isolation and characterization of rat cholangiocyte vesicles enriched in apical or basolateral plasma membrane domains. *Biochemistry* 1995; **34**: 15436-15443 [PMID: 7492544 DOI: 10.1021/bi00047a007]
59 **Ferjoux G**, Bousquet C, Cordelier P, Benali N, Lopez F, Rochaix P, Buscail L, Susini C. Signal transduction of somatostatin receptors negatively controlling cell proliferation. *J Physiol Paris* 2000; **94**: 205-210 [PMID: 11087998 DOI: 10.1016/S0928-4257(00)00206-0]
60 **Forrest JN**, Reichlin S, Goodman DB. Somatostatin: an endogenous peptide in the toad urinary bladder inhibits vasopressin-stimulated water flow. *Proc Natl Acad Sci USA* 1980; **77**: 4984-4987 [PMID: 6107910 DOI: 10.1073/pnas.77.8.4984]
61 **Friedlander G**, Amiel C. Somatostatin and alpha 2-adrenergic agonists selectively inhibit vasopressin-induced cyclic AMP accumulation in MDCK cells. *FEBS Lett* 1986; **198**: 38-42 [PMID: 2869974]
62 **Winkler SN**, Torikai S, Levine BS, Kurokawa K. Effect of somatostatin on vasopressin-induced antidiuresis and renal cyclic AMP of rats. *Miner Electrolyte Metab* 1982; **7**: 8-14 [PMID: 6133212]
63 **Mountokalakis T**, Levy M. Effect of somatostatin on renal water handling in the dog. *Can J Physiol Pharmacol* 1982; **60**: 655-664 [PMID: 6125252 DOI: 10.1139/y82-090]
64 **Pyronnet S**, Bousquet C, Najib S, Azar R, Laklai H, Susini C. Antitumor effects of somatostatin. *Mol Cell Endocrinol* 2008; **286**: 230-237 [PMID: 18359151 DOI: 10.1016/j.mce.2008.02.002]
65 **Bigg-Wither GW**, Ho KK, Grunstein RR, Sullivan CE, Doust BD. Effects of long term octreotide on gall stone formation and gall bladder function. *BMJ* 1992; **304**: 1611-1612 [PMID: 1628089 DOI: 10.1136/bmj.304.6842.1611]
66 **Jönsson A**, Manhem P. Octreotide and loss of scalp hair. *Ann Intern Med* 1991; **115**: 913 [PMID: 1952484 DOI: 10.7326/0003-4819-115-11-913_1]
67 **Kwekkeboom DJ**, Bakker WH, Kam BL, Teunissen JJ, Kooij PP, de Herder WW, Feelders RA, van Eijck CH, de Jong M, Srinivasan A, Erion JL, Krenning EP. Treatment of patients with gastro-entero-pancreatic (GEP) tumours with the novel radiolabelled somatostatin analogue [177Lu-DOTA(0),Tyr3]octreotate. *Eur J Nucl Med Mol Imaging* 2003; **30**: 417-422 [PMID: 12634971 DOI: 10.1007/s00259-002-1050-8]
68 **Lami MC**, Hadjadj S, Guillet G. Hair loss in three patients with acromegaly treated with octreotide. *Br J Dermatol* 2003; **149**: 655-656 [PMID: 14511007 DOI: 10.1046/j.1365-2133.2003.05478.x]
69 **Nakauchi Y**, Kumon Y, Yamasaki H, Tahara K, Kurisaka M, Hashimoto K. Scalp hair loss caused by octreotide in a patient with acromegaly: a case report. *Endocr J* 1995; **42**: 385-389 [PMID: 7670568 DOI: 10.1507/endocrj.42.385]
70 **Dilger JA**, Rho EH, Que FG, Sprung J. Octreotide-induced bradycardia and heart block during surgical resec-

tion of a carcinoid tumor. *Anesth Analg* 2004; **98**: 318-20, table of contents [PMID: 14742361 DOI: 10.1213/01.ANE.0000097170.27056.08]
71 **Herrington AM**, George KW, Moulds CC. Octreotide-induced bradycardia. *Pharmacotherapy* 1998; **18**: 413-416 [PMID: 9545165]
72 **Tzotzas T**, Papazisis K, Perros P, Krassas GE. Use of somatostatin analogues in obesity. *Drugs* 2008; **68**: 1963-1973 [PMID: 18778119 DOI: 10.2165/00003495-200868140-00003]
73 **Nakamura T**, Kudoh K, Takebe K, Imamura K, Terada A, Kikuchi H, Yamada N, Arai Y, Tando Y, Machida K. Octreotide decreases biliary and pancreatic exocrine function, and induces steatorrhea in healthy subjects. *Intern Med* 1994; **33**: 593-596 [PMID: 7827373 DOI: 10.2169/internalmedicine.33.593]
74 **Vachha B**, Sun MR, Siewert B, Eisenberg RL. Cystic lesions of the liver. *AJR Am J Roentgenol* 2011; **196**: W355-W366 [PMID: 21427297 DOI: 10.2214/AJR.10.5292]
75 **DeMatteo RP**, Lewis JJ, Leung D, Mudan SS, Woodruff JM, Brennan MF. Two hundred gastrointestinal stromal tumors: recurrence patterns and prognostic factors for survival. *Ann Surg* 2000; **231**: 51-58 [PMID: 10636102 DOI: 10.1097/00000658-200001000-00008]
76 **Hirota S**, Isozaki K, Moriyama Y, Hashimoto K, Nishida T, Ishiguro S, Kawano K, Hanada M, Kurata A, Takeda M, Muhammad Tunio G, Matsuzawa Y, Kanakura Y, Shinomura Y, Kitamura Y. Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. *Science* 1998; **279**: 577-580 [PMID: 9438854 DOI: 10.1126/science.279.5350.577]
77 **Ye YJ**, Gao ZD, Poston GJ, Wang S. Diagnosis and multidisciplinary management of hepatic metastases from gastrointestinal stromal tumour (GIST). *Eur J Surg Oncol* 2009; **35**: 787-792 [PMID: 19185444 DOI: 10.1016/j.ejso.2009.01.003]
78 **Matsuoka L**, Stapfer M, Mateo R, Jabbour N, Naing W, Selby R, Gagandeep S. Left extended hepatectomy for a metastatic gastrointestinal stromal tumor after a disease-free interval of 17 years: report of a case. *Surg Today* 2007; **37**: 70-73 [PMID: 17186351 DOI: 10.1007/s00595-006-3338-y]
79 **Radkani P**, Ghersi MM, Paramo JC, Mesko TW. A multidisciplinary approach for the treatment of GIST liver metastasis. *World J Surg Oncol* 2008; **6**: 46 [PMID: 18471285 DOI: 10.1186/1477-7819-6-46]
80 **Pisters PW**, Blanke CD, von Mehren M, Picus J, Sirulnik A, Stealey E, Trent JC. A USA registry of gastrointestinal stromal tumor patients: changes in practice over time and differences between community and academic practices. *Ann Oncol* 2011; **22**: 2523-2529 [PMID: 21464155 DOI: 10.1093/annonc/mdq773]
81 **Chen LL**, Trent JC, Wu EF, Fuller GN, Ramdas L, Zhang W, Raymond AK, Prieto VG, Oyedeji CO, Hunt KK, Pollock RE, Feig BW, Hayes KJ, Choi H, Macapinlac HA, Hittelman W, Velasco MA, Patel S, Burgess MA, Benjamin RS, Frazier ML. A missense mutation in KIT kinase domain 1 correlates with imatinib resistance in gastrointestinal stromal tumors. *Cancer Res* 2004; **64**: 5913-5919 [PMID: 15342366 DOI: 10.1158/0008-5472.CAN-04-0085]
82 **Heinrich MC**, Corless CL, Demetri GD, Blanke CD, von Mehren M, Joensuu H, McGreevey LS, Chen CJ, Van den Abbeele AD, Druker BJ, Kiese B, Eisenberg B, Roberts PJ, Singer S, Fletcher CD, Silberman S, Dimitrijevic S, Fletcher JA. Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. *J Clin Oncol* 2003; **21**: 4342-4349 [PMID: 14645423 DOI: 10.1200/JCO.2003.04.190]
83 **Heinrich MC**, Corless CL, Duensing A, McGreevey L, Chen CJ, Joseph N, Singer S, Griffith DJ, Haley A, Town A, Demetri GD, Fletcher CD, Fletcher JA. PDGFRA activating mutations in gastrointestinal stromal tumors. *Science* 2003; **299**: 708-710 [PMID: 12522257 DOI: 10.1126/science.1079666]
84 **Hirota S**, Ohashi A, Nishida T, Isozaki K, Kinoshita K, Shinomura Y, Kitamura Y. Gain-of-function mutations of platelet-derived growth factor receptor alpha gene in gastrointestinal stromal tumors. *Gastroenterology* 2003; **125**: 660-667 [PMID: 12949711 DOI: 10.1016/S0016-5085(03)01046-1]
85 **Chen P**, Zong L, Zhao W, Shi L. Efficacy evaluation of imatinib treatment in patients with gastrointestinal stromal tumors: a meta-analysis. *World J Gastroenterol* 2010; **16**: 4227-4232 [PMID: 20806443 DOI: 10.3748/wjg.v16.i33.4227]
86 **Gastrointestinal Stromal Tumor Meta-Analysis Group (MetaGIST)**. Comparison of two doses of imatinib for the treatment of unresectable or metastatic gastrointestinal stromal tumors: a meta-analysis of 1,640 patients. *J Clin Oncol* 2010; **28**: 1247-1253 [PMID: 20124181 DOI: 10.1200/JCO.2009.24.2099]
87 **Bümming P**, Andersson J, Meis-Kindblom JM, Klingenstierna H, Engström K, Stierner U, Wängberg B, Jansson S, Ahlman H, Kindblom LG, Nilsson B. Neoadjuvant, adjuvant and palliative treatment of gastrointestinal stromal tumours (GIST) with imatinib: a centre-based study of 17 patients. *Br J Cancer* 2003; **89**: 460-464 [PMID: 12888812 DOI: 10.1038/sj.bjc.6600965]
88 **Bauer S**, Hartmann JT, de Wit M, Lang H, Grabellus F, Antoch G, Niebel W, Erhard J, Ebeling P, Zeth M, Taeger G, Seeber S, Flasshove M, Schütte J. Resection of residual disease in patients with metastatic gastrointestinal stromal tumors responding to treatment with imatinib. *Int J Cancer* 2005; **117**: 316-325 [PMID: 15900603 DOI: 10.1002/ijc.21164]
89 **DeMatteo RP**, Maki RG, Singer S, Gonen M, Brennan MF, Antonescu CR. Results of tyrosine kinase inhibitor therapy followed by surgical resection for metastatic gastrointestinal stromal tumor. *Ann Surg* 2007; **245**: 347-352 [PMID: 17435539 DOI: 10.1097/01.sla.0000236630.93587.59]
90 **Raut CP**, Posner M, Desai J, Morgan JA, George S, Zahrieh D, Fletcher CD, Demetri GD, Bertagnolli MM. Surgical management of advanced gastrointestinal stromal tumors after treatment with targeted systemic therapy using kinase inhibitors. *J Clin Oncol* 2006; **24**: 2325-2331 [PMID: 16710031 DOI: 10.1200/JCO.2005.05.3439]
91 **Gronchi A**, Fiore M, Miselli F, Lagonigro MS, Coco P, Messina A, Pilotti S, Casali PG. Surgery of residual disease following molecular-targeted therapy with imatinib mesylate in advanced/metastatic GIST. *Ann Surg* 2007; **245**: 341-346 [PMID: 17435538 DOI: 10.1097/01.sla.0000242710.36384.1b]
92 **Suzuki S**, Sasajima K, Miyamoto M, Watanabe H, Yokoyama T, Maruyama H, Matsutani T, Liu A, Hosone M, Maeda S, Tajiri T. Pathologic complete response confirmed by surgical resection for liver metastases of gastrointestinal stromal tumor after treatment with imatinib mesylate. *World J Gastroenterol* 2008; **14**: 3763-3767 [PMID: 18595147 DOI: 10.3748/wjg.14.3763]
93 **Lendoire J**, Barros Schelotto P, Alvarez Rodríguez J, Duek F, Quarin C, Garay V, Amante M, Cassini E, Imventarza O. Bile duct cyst type V (Caroli's disease): surgical strategy and results. *HPB* (Oxford) 2007; **9**: 281-284 [PMID: 18345305 DOI: 10.1080/13651820701329258]
94 **Yonem O**, Bayraktar Y. Clinical characteristics of Caroli's disease. *World J Gastroenterol* 2007; **13**: 1930-1933 [PMID: 17461492]
95 **Shedda S**, Robertson A. Caroli's syndrome and adult polycystic kidney disease. *ANZ J Surg* 2007; **77**: 292-294 [PMID: 17388839 DOI: 10.1111/j.1445-2197.2006.03659.x]
96 **Wang ZX**, Yan LN, Li B, Zeng Y, Wen TF, Wang WT. Orthotopic liver transplantation for patients with Caroli's disease. *Hepatobiliary Pancreat Dis Int* 2008; **7**: 97-100 [PMID: 18234647]
97 **Kassahun WT**, Kahn T, Wittekind C, Mössner J, Caca K, Hauss J, Lamesch P. Caroli's disease: liver resection and liver transplantation. Experience in 33 patients. *Surgery* 2005; **138**: 888-898 [PMID: 16291390 DOI: 10.1016/j.surg.2005.05.002]

98 **Bockhorn M**, Malagó M, Lang H, Nadalin S, Paul A, Saner F, Frilling A, Broelsch CE. The role of surgery in Caroli's disease. *J Am Coll Surg* 2006; **202**: 928-932 [PMID: 16735207 DOI: 10.1016/j.jamcollsurg.2006.02.021]
99 **Xu HX**, Liu GJ, Lu MD, Xie XY, Xu ZF, Zheng YL, Liang JY. Characterization of small focal liver lesions using real-time contrast-enhanced sonography: diagnostic performance analysis in 200 patients. *J Ultrasound Med* 2006; **25**: 349-361 [PMID: 16495496]
100 **Xu HX**. Contrast-enhanced ultrasound in the biliary system: Potential uses and indications. *World J Radiol* 2009; **1**: 37-44 [PMID: 21160719 DOI: 10.4329/wjr.v1.i1.37]
101 **Inoue T**, Kitano M, Kudo M, Sakamoto H, Kawasaki T, Yasuda C, Maekawa K. Diagnosis of gallbladder diseases by contrast-enhanced phase-inversion harmonic ultrasonography. *Ultrasound Med Biol* 2007; **33**: 353-361 [PMID: 17280766 DOI: 10.1016/j.ultrasmedbio.2006.09.003]
102 **Numata K**, Oka H, Morimoto M, Sugimori K, Kunisaki R, Nihonmatsu H, Matsuo K, Nagano Y, Nozawa A, Tanaka K. Differential diagnosis of gallbladder diseases with contrast-enhanced harmonic gray scale ultrasonography. *J Ultrasound Med* 2007; **26**: 763-774 [PMID: 17526608]

P- Reviewers: Cardinale V, Xu HX **S- Editor:** Wen LL
L- Editor: Rutherford A **E- Editor:** Ma S

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v19.i43.7620

World J Gastroenterol 2013 November 21; 19(43): 7620-7629
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

REVIEW

Branched-chain amino acids in liver diseases

Kazuto Tajiri, Yukihiro Shimizu

Kazuto Tajiri, The Third Department of Internal Medicine, Toyama University Hospital, Toyama 930-0194, Japan
Yukihiro Shimizu, Gastroenterology Unit, Nanto Municipal Hospital, Toyama 932-0211, Japan
Author contributions: Tajiri K wrote the second half of the manuscript; and Shimizu Y wrote the first half of the manuscript and organized the whole manuscript.
Correspondence to: Yukihiro Shimizu, MD, PhD, Gastroenterology Unit, Nanto Municipal Hospital, Nanto, Toyama 932-0211, Japan. rsf14240@nifty.com
Telephone: +81-763-821475 Fax: +81-763-821853
Received: June 24, 2013 Revised: August 12, 2013
Accepted: September 15, 2013
Published online: November 21, 2013

Abstract

Branched chain amino acids (BCAAs) have been shown to affect gene expression, protein metabolism, apoptosis and regeneration of hepatocytes, and insulin resistance. They have also been shown to inhibit the proliferation of liver cancer cells *in vitro*, and are essential for lymphocyte proliferation and dendritic cell maturation. In patients with advanced chronic liver disease, BCAA concentrations are low, whereas the concentrations of aromatic amino acids such as phenylalanine and tyrosine are high, conditions that may be closely associated with hepatic encephalopathy and the prognosis of these patients. Based on these basic observations, patients with advanced chronic liver disease have been treated clinically with BCAA-rich medicines, with positive effects.

Key words: Liver disease; Branched chain amino acids; Gene expression; Hepatocyte apoptosis; Hepatocyte regeneration; Immunity; Treatment

Core tip: Advanced liver diseases are commonly accompanied by nutritional disturbances, which worsen the prognosis of the patients. Serum levels of branched-chain amino acids (BCAAs) are decreased in patients with liver cirrhosis, and the amino acids imbalance could affect the clinical picture of the disease and the prognosis of the patients. However, there are few comprehensive reviews on the biological activities of BCAAs. In this review, we summarize the biological activities of BCAAs, and discuss possible applications of BCAAs for the management of patients with advanced liver diseases with a list of clinical trials of BCAA administration.

Tajiri K, Shimizu Y. Branched-chain amino acids in liver diseases. *World J Gastroenterol* 2013; 19(43): 7620-7629 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7620.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7620

INTRODUCTION

The three branched chain amino acids (BCAAs), leucine, isoleucine and valine, are among the nine essential amino acids for humans. Recent studies have revealed the functions of these BCAAs, and they have been administered for the treatment of advanced liver diseases. In this review, we summarize current understanding of the biological properties of BCAAs and review the results of clinical application of BCAAs to treat patients with liver diseases.

BASIC ASPECTS OF BCAAS IN LIVER

Serum concentration of BCAAs in patients with chronic liver diseases and liver cirrhosis

Serum concentrations of BCAAs are decreased, while the concentrations of the aromatic amino acids (AAAs) phenylalanine and tyrosine are increased, in patients with advanced liver diseases, resulting in a low ratio of BCAAs to AAAs, a ratio called the Fischer ratio[1]. A low Fischer ratio has been associated with hepatic encephalopathy (HE). The imbalance of amino acids tends to become more marked with the progression of liver diseases, and aminograms are useful for assessing the prognosis of cir-

rhotic patients with or without hepatocellular carcinoma (HCC)[2,3]. Moreover, a simplified Fischer ratio, the BCAA to tyrosine ratio (BTR), has been reported useful for predicting serum albumin concentration one year later[4]. These data indicate that amino acid imbalance, either low Fischer ratio or BTR, is a marker for progression of liver diseases, and that correcting this ratio may have therapeutic potential, not only for nutritional improvement, but also for HE, in patients with advanced liver diseases.

Gene expression and mitochondrial biogenesis

In mice, BCAA-rich diets have shown to up-regulate the expression of peroxisome proliferator-activated receptor (PPAR) γ coactivator-1α (PGC-1α), a master regulator of mitochondrial biogenesis and the defense system against reactive oxygen species (ROS), and of sirtuin-1, a member of the sirtuin family linked to life span extension, enhanced mitochondrial biogenesis, and decreased ROS production, leading to the prolongation of the lifespan of male mice[5]. BCAAs have also been shown to induce the activation of genes involved in antioxidant defenses and inhibition of ROS production, as well as to induce the hepatic expression of mRNA encoding 8-oxyoguanine DNA glycosilase 1, an enzyme involved in repair of oxidative DNA damage, in a rat model of liver injury, indicating that BCAAs are involved in the induction of antioxidant DNA repair[6].

In various cell lines, BCAAs, especially leucine, have been shown to activate the mammalian target of rapamycin (mTOR) signals, stimulating the synthesis of proteins, including albumin, and of glycogen[7]. The ability of leucine to enhance glucose metabolism was confirmed in normal rats and in a rat cirrhosis model. BCAA activation of mTORC1 has also been associated with cell growth[8] and PGC-1α-mediated mitochondrial gene expression[9]. BCAAs have been shown to up-regulate PPAR-γ and uncouple (UCP) 2, reducing triglyceride concentrations in mouse livers[10]. These findings suggest that BCAAs may have a therapeutic effect on metabolic disorders and/or obesity.

Apoptosis and regeneration of hepatocytes

BCAA supplementation was shown to delay the progression of CCl_4-induced chronic liver injury in a rat model by reducing hepatic apoptosis[11]. On the other hand, BCAAs promoted hepatocyte regeneration in a rat model of hepatectomy[12]. Moreover, BCAAs were reported to stimulate the production of hepatocyte growth factor[13]. Taken together, these findings indicate that supplementation with BCAAs, by reducing hepatocyte apoptosis and promoting liver regeneration, may result in rapid recovery from liver injury.

Albumin synthesis

BCAAs activate mTOR and subsequently increase the production of eukaryotic initiation factor 4E-binding protein-1 and ribosomal protein S6 kinase, which upregulate the synthesis of albumin[14-16]. Furthermore, leucine stimulates the nuclear importation of polypyrimidine-tract-binding protein, which binds to albumin mRNA and increases its translation[17].

Insulin resistance

BCAAs were shown to improve homeostasis model assessment scores for insulin resistance (HOMA-IR) and beta cell function (HOMA-%B) in patients with chronic liver disease, indicating that BCAAs can ameliorate insulin resistance[18]. In mice lacking the gene encoding mitochondrial BCAA aminotransferase, an enzyme that catalyzes BCAAs, serum BCAA concentrations were elevated. In those mice, fasting blood glucose and insulin concentrations were decreased and HOMA-IR was significantly lower than in wild-type mice[19]. Furthermore, administration of leucine or isoleucine improved insulin sensitivity in mice with high-fat diets[20,21]. BCAAs were also shown to temporarily increase plasma insulin concentrations in healthy young men, although plasma glucose concentrations were not altered[22].

Several organs are involved in the mechanism by which BCAAs improve insulin resistance. In the liver, BCAAs increase the liver X receptor/sterol regulatory element binding protein-1c pathway and subsequently activate liver-type glucokinase and glucose transporter. Furthermore, BCAAs suppress hepatic expression of glucose-6-phosphatase[23]. In adipose tissue, leucine increases insulin-induced phosphorylation of Akt and mTOR, increasing glucose uptake[24]. In skeletal muscle, BCAAs promote glucose uptake through activation of phosphatidylinositol 3-kinase (PI3K) and protein kinase C and subsequent translocation of glucose transporter to the plasma membrane[25]. In addition, BCAAs increase PPAR-γ and subsequent UCP2 in liver and UCP3 in muscle, stimulating oxidation of free fatty acids. Thus, BCAAs improve insulin resistance through interactions in organs targeted by insulin.

Liver cancer cells

The direct effects of BCAAs on liver cancer cells have been analyzed in culture systems. Increased concentrations of BCAAs in culture medium were reported to suppress the proliferation of HCC cell lines[26]. Moreover, all three BCAAs were found to accelerate insulin-induced vascular endothelial growth factor (VEGF) mRNA degradation at the post transcriptional level, downregulating VEGF expression during the development of HCCs[27]. BCAAs were also shown to induce apoptosis of liver cancer cell lines by inhibiting insulin-induced PI3K/Akt and NFκB pathways through mTORC1- and mTORC2-dependent mechanisms[28]. Moreover, BCAAs may inhibit obesity-related hepatocarcinogenesis by suppressing the stimulatory effect of visfatin, an adipokine with a critical role in HCC proliferation[29].

Insulin was found to induce cell proliferation through activation of the mitogen-activated protein kinase pathway[30], and BCAAs inhibit insulin signals by suppressing the expression of insulin-like growth factor[31]. BCAAs have been reported to decrease insulin resistance-induced

expression of endothelial growth factor and to subsequently suppress tumor angiogenesis[32]. Collectively, these data suggest that BCAAs inhibit the proliferation of HCC cells or hepatocarcinogenesis through multiple mechanisms.

Immunity

Immunity and nutrition are closely associated, and several studies have indicated the importance of BCAAs during lymphocyte proliferation or dendritic cell maturation. Depletion of any of the three BCAAs from the culture medium was shown to markedly inhibit phytohemagglutinin-induced lymphocyte proliferation[33], with removal of valine from the culture medium completely abolishing lymphocyte proliferation. In contrast, increased concentrations of BCAAs in the culture medium did not significantly affect lymphocyte proliferation, indicating that, although the BCAAs are requisite for lymphocyte proliferation, there are optimal concentrations. On the other hand, BCAAs have little effect on macrophage functions.

In vivo studies have also shown the importance of BCAAs for immunity. We previously analyzed the effects of a BCAA-rich diet on immune system functions in the spleen and liver of rats[34]. We found that addition of BCAAs to the diet increased the numbers of intrahepatic lymphocytes and stimulated natural killer (NK) cell activity and lectin-dependent cytotoxic activities in the liver. Interestingly, the number of intrahepatic lymphocytes was positively correlated with valine concentrations in plasma and the liver. BCAAs, especially valine, are also involved in the maturation of dendritic cells. For example, valine was found to dose-dependently increase the allostimulatory capacity of IL-12 production by monocyte-derived dendritic cells (DCs) obtained from both healthy volunteers and cirrhotic patients with chronic hepatitis C virus (HCV) infection[35]. These findings suggest that valine may have therapeutic potential in HCV-infected cirrhotic patients by restoring immune system activities, which may lead to inhibit hepatocarcinogenesis[35,36]. In patients with cirrhosis, BCAA administration increases the numbers of hepatic lymphocytes and restores the phagocytic activity of neutrophils and the NK activity of lymphocytes[37]. In addition, BCAAs increased the number of blood lymphocytes in postsurgical patients[38,39], and significant correlations were observed between the serum concentration of BCAAs and the survival rates of the patients with sepsis[40]. These data indicate that BCAAs are closely associated with the maturation and function of various immune cells.

CLINICAL APPLICATION OF BCAAS IN LIVER DISEASES

BCAAs for liver cirrhosis

The liver is a central organ for nutrient metabolism, and patients with chronic liver diseases may develop various metabolic and nutrition disorders[41]. Patients with cirrhosis frequently show protein and energy deficiency. Protein deficiency leads to hypoalbuminemia, inducing ascites and edema, whereas energy deficiency decreases fat and muscle mass and causes muscle weakness, decreasing the quality of life of patients with cirrhosis[42]. Several clinical trials have suggested that BCAA supplementation improves the prognosis of cirrhotic patients[43,44]. For example, a multicenter randomized trial from Italy showed that oral BCAA supplementation in patients with advanced cirrhosis prevented progressive hepatic failure and improved surrogate markers and perceived health status[44]. Furthermore, a large scale post marketing clinical study in Japan showed that oral BCAA administration significantly reduced the occurrence of complications associated with poor prognosis, such as liver failure, ruptured esophageal varices, HCC, and death, compared with patients who received diet therapy with defined daily food intake (HR = 0.67, 95%CI: 0.49-0.93)[43]. Furthermore, BCAA supplementation in patients with advanced cirrhosis may improve abnormal glucose tolerance in addition to improving serum albumin concentration[45], and a randomized study showed that oral BCAA was effective in patients with both compensated and decompensated cirrhosis, maintaining or increasing serum albumin concentrations[46]. Oral BCAA treatment has also been reported to improve protein malnutrition in patients, especially during the early stages of liver cirrhosis, increasing serum albumin level to 3.5-3.9 g/dL and increasing total hepatic parenchymal cell mass[47-49]. BCAA treatment also improved nutritional status and reduced the frequency of albumin infusion in children with end-stage liver disease[50]. Taken together, these findings indicate that BCAA supplementation is effective in improving nutritional status in cirrhotic patients, regardless of patient age or disease stage.

Furthermore, BCAA supplementation was reported to improve the quality of life in cirrhotic patients. Two randomized trials showed that BCAA supplementation improved the Short Form-36 scores of general health perception compared with control groups[43,44]. Another randomized study showed that BCAA-enriched supplements improved weakness and fatigue compared with ordinary foods[51]. BCAA-enriched supplementation has also been reported to improve sleep disturbance[52].

Accelerated fat oxidation and a catabolic state after fasting, represented as a decreased respiratory quotient (RQ), are frequently observed in patients with cirrhosis[53]. Late evening snack supplementation with a BCAA mixture was found to improve RQ, nutritional state and glucose intolerance[53,54]. The energy efficiency of BCAAs is higher than that of glucose or fatty acids, suggesting that BCAAs may be the preferred energy substrate for patients with cirrhosis[55]. Others also reported that late evening snacks with BCAAs were useful in improving protein metabolism and lipolysis in cirrhotic patients[56].

Thus, BCAA supplementation for advanced cirrhotic patients improves nutritional status and quality of life. The guidelines of the European Society for Clinical Nutrition and Metabolism and the Study Group for the Standardization of Treatment of Viral Hepatitis Includ-

ing Cirrhosis of the Ministry of Health, Labour and Welfare of Japan recommend BCAA supplementation in the treatment of patients with advanced cirrhosis[57,58].

BCAAs for hepatic encephalopathy

HE is a major complication of cirrhosis associated with poor prognosis and quality of life, and often occurs repeatedly. Elevated blood ammonia is seen in patients with HE, and ammonia is one of the pathogenic factors for the development of HE[59]. Unfortunately, infusion of BCAAs was reported to increase venous blood ammonia in most patients with liver failure[60]. Thus, the effects of BCAAs on HE may not be associated with blood ammonia levels, especially when administered intravenously. HE may also be caused by a decreased plasma ratio of BCAAs to AAAs. In patients with advanced cirrhosis, HE frequently occurs after gastrointestinal bleeding, perhaps due to an absence of isoleucine and an abundance of leucine in hemoglobin molecules, leading to HE by way of BCAA antagonism[61]. Treatment with BCAAs may therefore have a beneficial effect on patients with hepatic encephalopathy mainly by compensating decreased ratio of BCAAs to AAAs, but not by reducing serum ammonia levels. A systematic review reported that BCAAs appeared to have a modest effect in improving encephalopathy without adverse events, although convincing evidence was not supplied[62]. Two randomized studies also showed that BCAAs did not clearly prevent HE in patients with advanced cirrhosis, although BCAAs prevented the progression of hepatic failure[43,44]. Furthermore, postoperative BCAA treatment could not prevent postoperative hepatic encephalopathy[63]. A recent randomized, double-blind, multicenter study evaluating the effect of BCAAs on HE found that BCAAs did not decrease the recurrence of HE but improved minimal HE and muscle mass[64]. Moreover, a systematic review showed that oral (RR = 1.44; 95%CI: 1.07-1.94) but not intravenous (RR=1.12; 95%CI: 0.91-1.39) administration of BCAAs improved HE manifestations[65]. Non-absorbable disaccharides such as lactulose or lactitol also improved the manifestations of HE (RR = 1.99; 95%CI: 1.14-3.48) and prevented clinically overt HE (RR = 0.26; 95%CI: 0.17-0.41), suggesting that non-absorbable disaccharides be used as the first line treatment of HE and BCAAs may be considered as a second line treatment[65].

Recently, a systematic review with meta-analyses on the effect of oral BCAAs for the treatment of HE was published[66]. The review has revealed that supplementation of oral BCAAs in cirrhotic patients inhibits the manifestation of HE, especially in patients with overt HE rather than those with minimal HE, but showed no effect on the survival of those patients[66]. Thus, oral administration of BCAAs is the treatment of choice in cirrhotic patients with HE, especially in combination with non-absorbable disaccharides.

BCAAs for hepatocellular carcinoma

Clinical studies have suggested that BCAA supplementation can help in the management of HCC. Prolonged surgical stress and advanced malignancy can result in systemic catabolism and muscle wasting, with BCAA supplementation having the potential to improve these conditions[67].

A randomized control trial in obese, HCV-infected patients with cirrhosis showed that BCAA supplementation reduced the frequency of development of HCC, by approximately 30% over 3 years[68]. In addition, a second randomized trial in patients with compensated liver cirrhosis due to HCV showed that oral BCAAs reduced the incidence of HCC (15.8% *vs* 25.0%)[69]. A retrospective analysis in patients with cirrhosis showed that the incidence of HCC was significantly lower in patients who did than did not receive BCAAs (HR = 0.416, 95%CI: 0.216-0.800, P = 0.0085) [70]. Furthermore, combinations of BCAAs and angiotensin-converting enzyme inhibitors may prevent the development of HCC in patients with insulin resistance[71].

Perioperative nutritional support, especially enteral rather than parental nutrition, was found to improve the prognosis of cirrhotic patients by reducing complications following hepatectomy[72,73]. Recently, a randomized trial showed that BCAA supplementation after hepatectomy promoted rapid improvement in protein metabolism and inhibited progression to liver cirrhosis[74]. Furthermore, another randomized trial showed that oral BCAA supplementation after hepatectomy for HCC significantly reduced the 30 month recurrence of HCC (28.5% *vs* 55.7%, P = 0.044)[75]. Perioperative BCAA treatment in patients undergoing hepatectomy was also shown to contribute to shorter hospital stay and quicker improvement of liver function during the early postoperative period[76] and to improve postoperative quality of life by restoring and maintaining nutritional status and whole-body kinetics[77].

The effect of BCAAs on HCC recurrence after radiofrequency ablation (RFA) remains unclear. Two prospective studies showed that BCAA supplementation improved nutritional state and liver function, but its effect on HCC recurrence was not determined[78,79]. However, a recent retrospective study showed that oral BCAA supplementation after RFA improved 1 year (61.8% *vs* 52.0%) and 3-year (28.0% *vs* 12.0%) progression-free survival rates compared with a control group after RFA (P = 0.013) [80].

Oral BCAA supplementation after chemoembolization also prevents the decrease of liver function after treatment and improves the quality of life, although its ability to prevent HCC recurrence was not determined[81,82]. Oral BCAA treatment before chemoembolization was found useful in maintaining hepatic functional reserve[83]. A randomized trial also found that oral BCAA supplementation improved nutritional status by increasing BCAA concentration during radiotherapy for HCC[84].

Thus, BCAA supplementation for patients with HCC is of clinical importance in the preservation of liver function and quality of life during treatment, although it is unclear whether BCCAs directly prevent HCC.

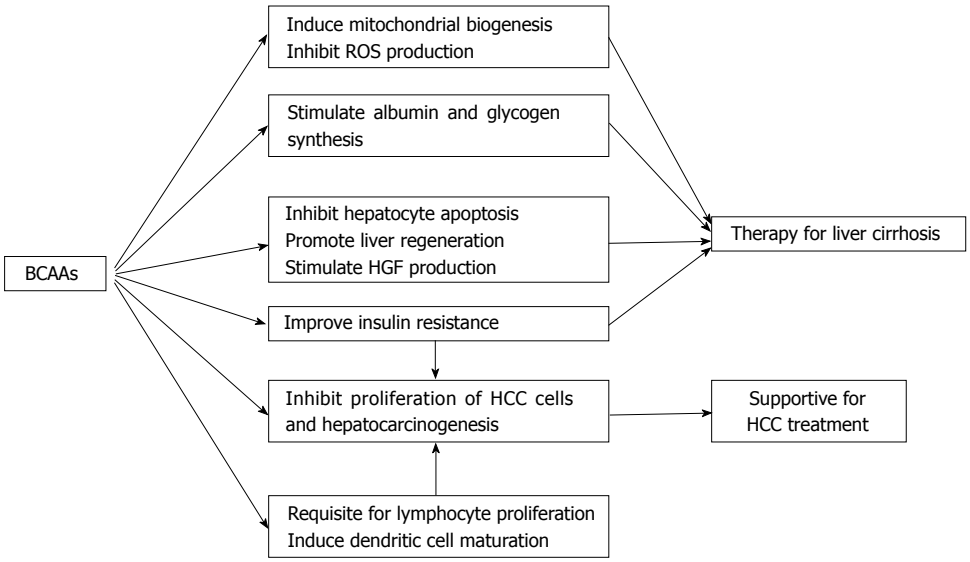


Figure 1 Mechanism of action of branched chain amino acids in liver diseases. BCAAs: Branched chain amino acids; ROS: Reactive oxygen species; HGF: Hepatocyte growth factor; HCC: Hepatocellular carcinoma.

Table 1 Prospective randomized trials of branched-chain amino acid administration for advanced liver diseases

Object	Time	No.	Major outcome	Ref.
Cirrhosis	2 yr	646	Improve event-free survival and QOL. Increase serum albumin levels.	[43]
Cirrhosis (advanced)	1 yr	174	Improve event-free survival. Lower hospital admission. Improve the Child-Pugh score and QOL.	[44]
Cirrhosis (decompensated)	24 wk	281	Increase serum albumin levels.	[45]
Cirrhosis	2 yr	65	Maintain serum albumin levels.	[46]
Cirrhosis (early)	2 yr	65	Maintain serum albumin levels.	[49]
Cirrhosis	3 mo	48	Increase serum albumin levels. Improve energy metabolism.	[51]
Cirrhosis (HCV)	168 wk	39	Reduce hepatic carcinogenesis in patients with compensated cirrhosis with a serum albumin level of < 4.0 g/dL.	[69]
Cirrhosis (HCV, obese)	2 yr	622	Reduce hepatic carcinogenesis in patients with BMI of 25 or higher and with an alpha-fetoprotein level of 20 ng/mL or higher.	[68]
Cirrhosis (pre liver transplant)	3.3 yr	50	Preserve hepatic reserve functions. Lower complications associated with cirrhosis.	[99]
Cirrhosis after an episode of HE	56 wk	116	Not decrease recurrence of HE. Improve minimal HE and muscle mass.	[64]
Cirrhosis after hepatectomy	1 yr	43	Improve hepatic metabolism after hepatectomy. Inhibit progression to cirrhosis.	[74]
HCC after hepatectomy	6.5 mo	56	Reduce early recurrence of HCC.	[75]
HCC after hepatectomy	12 wk	44	Shorten hospital stay. Quicker improvement of liver functions.	[76]
After hepatectomy	12 mo	76	Improve post operative QOL.	[77]
HCC after RFA	12 mo	35	Improve nutritional state and QOL.	[78]
HCC after RFA	12 wk	30	Improve liver functions.	[79]
HCC undergoing chemoembolization	12 mo	84	Increase serum albumin levels, reduce morbidity, and improve QOL.	[81]
HCC undergoing chemoembolization	2 wk	56	Prevent reduction of liver functions.	[82]
HCC during radiotherapy	10 wk	30	Increase serum albumin levels.	[84]

QOL: Quality of life; HCV: Hepatitis C virus; HE: Hepatic encephalopathy; HCC: Hepatocellular caricinoma. RFA: Radiofrequency ablation.

Acute liver injury

Although BCAAs have no proven benefit in patients with acute liver injury, enteric nutritional support is essential[85]. Several animal studies have shown that BCAAs may prevent acute liver injury[86-88], although its effects in humans are as yet undetermined. BCAA concentrations have been reported to be increased, unaltered or decreased following acute liver injury[89,90]. In alcoholic hepatitis, parentally or enterally administered hyperalimentation with or without BCAAs did not show survival benefits[91].

HCV infection

Insulin resistance occurs frequently in patients infected with HCV and is associated with various complications, such as steatosis, disturbances in glucose metabolism, and carcinogenesis[92]. BCAAs, especially leucine or isoleucine, have been shown to have beneficial effects on glucose metabolism[93]. A randomized study showed that BCAA treatment of patients with chronic hepatitis C and insulin resistance improved HbA1c concentrations in patients with marked peripheral insulin resistance, although

BCAA did not significantly affect parameters of glucose metabolism or lipid profiles[94]. A multicenter randomized control trial showed that BCAAs prevented the development of HCC in obese, HCV-infected patients[68]. Furthermore, BCAA treatment can restore impaired interferon signaling caused by malnutrition through the mTOR and FoxO pathways in patients with chronic hepatitis C[95]. Interestingly, valine was reported to reduce HCV viral load, possibly by enhancing DC function or interferon signaling[96]. Thus, BCAA supplementation may be useful for adherence to interferon therapy in patients with chronic hepatitis C and may enhance the effects of interferon in these patients[97].

Liver transplantation

Protein-energy malnutrition is commonly found in patients with end-stage liver disease requiring liver transplantation and is a risk factor for posttransplant morbidity. A report of 50 recipients undergoing living donor liver transplantation (LDLT) showed that absence of preoperative BCAA treatment was an independent risk factor for postoperative severe infection and in-hospital death[98]. Kawamura *et al*[99] reported that early interventional oral BCAAs might prolong the liver transplant waiting period by preserving hepatic reserve in patients with cirrhosis. A retrospective analysis also showed that BCAA treatment before LDLT may reduce the incidence of posttransplant bacteremia[100].

Other clinical problems related to management of liver diseases

Insulin resistance: Increased insulin resistance is found in patients with chronic liver diseases and is a therapeutic target associated with malnutrition and hepatocarcinogenesis. BCAAs are thought to act on insulin target organs, such as skeletal muscles, adipose tissue, and the liver[101]. BCAA infusion was reported to decrease plasma glucose concentrations in patients with advanced liver cirrhosis[102], and oral BCAA administration was recently shown to reduce both blood glucose concentrations[103,104] and insulin resistance in patients with chronic liver diseases, especially in men[19,105]. More recently, long-term BCAA supplementation was shown to improve glucose tolerance in patients with nonalcoholic steatohepatitis (NASH)-related cirrhosis, and may be an alternative treatment for NASH[106].

CONCLUSION

BCAAs are involved in various biological activities (Figure 1), and prospective randomized clinical trials showing possible effectiveness of BCAAs in the management of chronic liver diseases are summarized in Table 1. Supplementation with BCAAs may be a promising therapeutic option for patients with chronic liver diseases, although more analyses are needed to determine their basic mechanisms of action.

REFERENCES

1 **Campollo O**, Sprengers D, McIntyre N. The BCAA/AAA ratio of plasma amino acids in three different groups of cirrhotics. *Rev Invest Clin* 1992; **44**: 513-518 [PMID: 1485030]

2 **Steigmann F**, Szanto PB, Poulos A, Lim PE, Dubin A. Significance of serum aminograms in diagnosis and prognosis of liver diseases. *J Clin Gastroenterol* 1984; **6**: 453-460 [PMID: 6501833]

3 **Watanabe A**, Higashi T, Sakata T, Nagashima H. Serum amino acid levels in patients with hepatocellular carcinoma. *Cancer* 1984; **54**: 1875-1882 [PMID: 6090002]

4 **Suzuki K**, Suzuki K, Koizumi K, Ichimura H, Oka S, Takada H, Kuwayama H. Measurement of serum branched-chain amino acids to tyrosine ratio level is useful in a prediction of a change of serum albumin level in chronic liver disease. *Hepatol Res* 2008; **38**: 267-272 [PMID: 18021234]

5 **D'Antona G**, Ragni M, Cardile A, Tedesco L, Dossena M, Bruttini F, Caliaro F, Corsetti G, Bottinelli R, Carruba MO, Valerio A, Nisoli E. Branched-chain amino acid supplementation promotes survival and supports cardiac and skeletal muscle mitochondrial biogenesis in middle-aged mice. *Cell Metab* 2010; **12**: 362-372 [PMID: 20889128 DOI: 10.1016/j.cmet.2010.08.016]

6 **Ichikawa K**, Okabayashi T, Shima Y, Iiyama T, Takezaki Y, Munekage M, Namikawa T, Sugimoto T, Kobayashi M, Mimura T, Hanazaki K. Branched-chain amino acid-enriched nutrients stimulate antioxidant DNA repair in a rat model of liver injury induced by carbon tetrachloride. *Mol Biol Rep* 2012; **39**: 10803-10810 [PMID: 23053977 DOI: 10.1007/s11033-012-1974-4]

7 **Nishitani S**, Ijichi C, Takehana K, Fujitani S, Sonaka I. Pharmacological activities of branched-chain amino acids: specificity of tissue and signal transduction. *Biochem Biophys Res Commun* 2004; **313**: 387-389 [PMID: 14684173]

8 **Schieke SM**, Phillips D, McCoy JP, Aponte AM, Shen RF, Balaban RS, Finkel T. The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity. *J Biol Chem* 2006; **281**: 27643-27652 [PMID: 16847060]

9 **Cunningham JT**, Rodgers JT, Arlow DH, Vazquez F, Mootha VK, Puigserver P. mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. *Nature* 2007; **450**: 736-740 [PMID: 18046414]

10 **Arakawa M**, Masaki T, Nishimura J, Seike M, Yoshimatsu H. The effects of branched-chain amino acid granules on the accumulation of tissue triglycerides and uncoupling proteins in diet-induced obese mice. *Endocr J* 2011; **58**: 161-170 [PMID: 21372430]

11 **Kuwahata M**, Kubota H, Kanouchi H, Ito S, Ogawa A, Kobayashi Y, Kido Y. Supplementation with branched-chain amino acids attenuates hepatic apoptosis in rats with chronic liver disease. *Nutr Res* 2012; **32**: 522-529 [PMID: 22901560 DOI: 10.1016/j.nutres.2012.06.007]

12 **Kim SJ**, Kim DG, Lee MD. Effects of branched-chain amino acid infusions on liver regeneration and plasma amino acid patterns in partially hepatectomized rats. *Hepatogastroenterology* 2011; **58**: 1280-1285 [PMID: 21937393]

13 **Tomiya T**, Omata M, Fujiwara K. Significance of branched chain amino acids as possible stimulators of hepatocyte growth factor. *Biochem Biophys Res Commun* 2004; **313**: 411-416 [PMID: 14684177 DOI: 10.1016/j.bbrc.2003.07.017]

14 **Okuno M**, Moriwaki H, Kato M, Muto Y, Kojima S. Changes in the ratio of branched-chain to aromatic amino acids affect the secretion of albumin in cultured rat hepatocytes. *Biochem Biophys Res Commun* 1995; **214**: 1045-1050 [PMID: 7575508 DOI: 10.1006/bbrc.1995.2391]

15 **Ijichi C**, Matsumura T, Tsuji T, Eto Y. Branched-chain amino acids promote albumin synthesis in rat primary hepatocytes through the mTOR signal transduction system. *Biochem Biophys Res Commun* 2003; **303**: 59-64 [PMID: 12646166 DOI: 10.1016/S0006-291X(03)00295-X]

16 **Montoya A**, Gómez-Lechón MJ, Castell JV. Influence of

branched-chain amino acid composition of culture media on the synthesis of plasma proteins by serum-free cultured rat hepatocytes. *In Vitro Cell Dev Biol* 1989; **25**: 358-364 [PMID: 2715129 DOI: 10.1007/BF02624599]

17 **Kuwahata M**, Yoshimura T, Sawai Y, Amano S, Tomoe Y, Segawa H, Tatsumi S, Ito M, Ishizaki S, Ijichi C, Sonaka I, Oka T, Miyamoto K. Localization of polypyrimidine-tract-binding protein is involved in the regulation of albumin synthesis by branched-chain amino acids in HepG2 cells. *J Nutr Biochem* 2008; **19**: 438-447 [PMID: 17707630 DOI: 10.1016/j.jnutbio.2007.05.011]

18 **Kawaguchi T**, Nagao Y, Matsuoka H, Ide T, Sata M. Branched-chain amino acid-enriched supplementation improves insulin resistance in patients with chronic liver disease. *Int J Mol Med* 2008; **22**: 105-112 [PMID: 18575782]

19 **She P**, Reid TM, Bronson SK, Vary TC, Hajnal A, Lynch CJ, Hutson SM. Disruption of BCATm in mice leads to increased energy expenditure associated with the activation of a futile protein turnover cycle. *Cell Metab* 2007; **6**: 181-194 [PMID: 17767905 DOI: 10.1016/j.cmet.2007.08.003]

20 **Ikehara O**, Kawasaki N, Maezono K, Komatsu M, Konishi A. Acute and chronic treatment of L-isoleucine ameliorates glucose metabolism in glucose-intolerant and diabetic mice. *Biol Pharm Bull* 2008; **31**: 469-472 [PMID: 18310912 DOI: 10.1248/bpb.31.469]

21 **Zhang Y**, Guo K, LeBlanc RE, Loh D, Schwartz GJ, Yu YH. Increasing dietary leucine intake reduces diet-induced obesity and improves glucose and cholesterol metabolism in mice via multimechanisms. *Diabetes* 2007; **56**: 1647-1654 [PMID: 17360978 DOI: 10.2337/db07-0123]

22 **Zhang Y**, Kobayashi H, Mawatari K, Sato J, Bajotto G, Kitaura Y, Shimomura Y. Effects of branched-chain amino acid supplementation on plasma concentrations of free amino acids, insulin, and energy substrates in young men. *J Nutr Sci Vitaminol* (Tokyo) 2011; **57**: 114-117 [PMID: 21512300 DOI: 10.3177/jnsv.57.114]

23 **Higuchi N**, Kato M, Miyazaki M, Tanaka M, Kohjima M, Ito T, Nakamuta M, Enjoji M, Kotoh K, Takayanagi R. Potential role of branched-chain amino acids in glucose metabolism through the accelerated induction of the glucose-sensing apparatus in the liver. *J Cell Biochem* 2011; **112**: 30-38 [PMID: 20506195 DOI: 10.1002/jcb.22688]

24 **Hinault C**, Mothe-Satney I, Gautier N, Lawrence JC, Van Obberghen E. Amino acids and leucine allow insulin activation of the PKB/mTOR pathway in normal adipocytes treated with wortmannin and in adipocytes from db/db mice. *FASEB J* 2004; **18**: 1894-1896 [PMID: 15479767]

25 **Nishitani S**, Matsumura T, Fujitani S, Sonaka I, Miura Y, Yagasaki K. Leucine promotes glucose uptake in skeletal muscles of rats. *Biochem Biophys Res Commun* 2002; **299**: 693-696 [PMID: 12470633 DOI: 10.1016/S0006-291X(02)02717-1]

26 **Sugiyama K**, Yu L, Nagasue N. Direct effect of branched-chain amino acids on the growth and metabolism of cultured human hepatocellular carcinoma cells. *Nutr Cancer* 1998; **31**: 62-68 [PMID: 9682250 DOI: 10.1080/01635589809514679]

27 **Miuma S**, Ichikawa T, Arima K, Takeshita S, Muraoka T, Matsuzaki T, Ootani M, Shibata H, Akiyama M, Ozawa E, Miyaaki H, Taura N, Takeshima F, Nakao K. Branched-chain amino acid deficiency stabilizes insulin-induced vascular endothelial growth factor mRNA in hepatocellular carcinoma cells. *J Cell Biochem* 2012; **113**: 3113-3121 [PMID: 22581719 DOI: 10.1002/jcb.24188]

28 **Hagiwara A**, Nishiyama M, Ishizaki S. Branched-chain amino acids prevent insulin-induced hepatic tumor cell proliferation by inducing apoptosis through mTORC1 and mTORC2-dependent mechanisms. *J Cell Physiol* 2012; **227**: 2097-2105 [PMID: 21769869 DOI: 10.1002/jcp.22941]

29 **Ninomiya S**, Shimizu M, Imai K, Takai K, Shiraki M, Hara T, Tsurumi H, Ishizaki S, Moriwaki H. Possible role of visfatin in hepatoma progression and the effects of branched-chain amino acids on visfatin-induced proliferation in human hepatoma cells. *Cancer Prev Res* (Phila) 2011; **4**: 2092-2100 [PMID: 21952585 DOI: 10.1158/1940-6207.CAPR-11-0340]

30 **Formisano P**, Oriente F, Fiory F, Caruso M, Miele C, Maitan MA, Andreozzi F, Vigliotta G, Condorelli G, Beguinot F. Insulin-activated protein kinase Cbeta bypasses Ras and stimulates mitogen-activated protein kinase activity and cell proliferation in muscle cells. *Mol Cell Biol* 2000; **20**: 6323-6333 [PMID: 10938109 DOI: 10.1128/MCB.20.17.6323-6333.2000]

31 **Iwasa J**, Shimizu M, Shiraki M, Shirakami Y, Sakai H, Terakura Y, Takai K, Tsurumi H, Tanaka T, Moriwaki H. Dietary supplementation with branched-chain amino acids suppresses diethylnitrosamine-induced liver tumorigenesis in obese and diabetic C57BL/KsJ-db/db mice. *Cancer Sci* 2010; **101**: 460-467 [PMID: 19906067 DOI: 10.1111/j.1349-7006.2009.01402.x]

32 **Yoshiji H**, Noguchi R, Kaji K, Ikenaka Y, Shirai Y, Namisaki T, Kitade M, Tsujimoto T, Kawaratani H, Fukui H. Attenuation of insulin-resistance-based hepatocarcinogenesis and angiogenesis by combined treatment with branched-chain amino acids and angiotensin-converting enzyme inhibitor in obese diabetic rats. *J Gastroenterol* 2010; **45**: 443-450 [PMID: 19941011 DOI: 10.1007/s00535-009-0158-z]

33 **Chuang JC**, Yu CL, Wang SR. Modulation of lymphocyte proliferation by enzymes that degrade amino acids. *Clin Exp Immunol* 1990; **82**: 469-472 [PMID: 2124955]

34 **Tsukishiro T**, Shimizu Y, Higuchi K, Watanabe A. Effect of branched-chain amino acids on the composition and cytolytic activity of liver-associated lymphocytes in rats. *J Gastroenterol Hepatol* 2000; **15**: 849-859 [PMID: 11022824 DOI: 10.1046/j.1440-1746.2000.02220.x]

35 **Kakazu E**, Kanno N, Ueno Y, Shimosegawa T. Extracellular branched-chain amino acids, especially valine, regulate maturation and function of monocyte-derived dendritic cells. *J Immunol* 2007; **179**: 7137-7146 [PMID: 17982106]

36 **Kakazu E**, Ueno Y, Kondo Y, Fukushima K, Shiina M, Inoue J, Tamai K, Ninomiya M, Shimosegawa T. Branched chain amino acids enhance the maturation and function of myeloid dendritic cells ex vivo in patients with advanced cirrhosis. *Hepatology* 2009; **50**: 1936-1945 [PMID: 19885880 DOI: 10.1002/hep.23248]

37 **Nakamura I**, Ochiai K, Imai Y, Moriyasu F, Imawari M. Restoration of innate host defense responses by oral supplementation of branched-chain amino acids in decompensated cirrhotic patients. *Hepatol Res* 2007; **37**: 1062-1067 [PMID: 17608669 DOI: 10.1111/j.1872-034X.2007.00166.x]

38 **Nuwer N**, Cerra FB, Shronts EP, Lysne J, Teasley KM, Konstantinides FN. Does modified amino acid total parenteral nutrition alter immune-response in high level surgical stress. *JPEN J Parenter Enteral Nutr* 1983; **7**: 521-524 [PMID: 6418905 DOI: 10.1177/014860718300700652]

39 **Cerra FB**, Mazuski JE, Chute E, Nuwer N, Teasley K, Lysne J, Shronts EP, Konstantinides FN. Branched chain metabolic support. A prospective, randomized, double-blind trial in surgical stress. *Ann Surg* 1984; **199**: 286-291 [PMID: 6422868 DOI: 10.1097/00000658-198403000-00007]

40 **De Bandt JP**, Cynober L. Therapeutic use of branched-chain amino acids in burn, trauma, and sepsis. *J Nutr* 2006; **136**: 308S-313S [PMID: 16365104]

41 **Charlton MR**. Protein metabolism and liver disease. *Baillieres Clin Endocrinol Metab* 1996; **10**: 617-635 [PMID: 9022955 DOI: 10.1016/S0950-351X(96)80771-3]

42 **Miwa Y**, Moriwaki H. Nocturnal energy and BCAA supplementation in patients with liver cirrhosis. *Hepatol Res* 2004; **30S**: 63-66 [PMID: 15607141 DOI: 10.1016/j.hepres.2004.08.012]

43 **Muto Y**, Sato S, Watanabe A, Moriwaki H, Suzuki K, Kato A, Kato M, Nakamura T, Higuchi K, Nishiguchi S, Kumada H. Effects of oral branched-chain amino acid granules on event-free survival in patients with liver cirrhosis. *Clin Gastroen-*

terol Hepatol 2005; **3**: 705-713 [PMID: 16206505 DOI: 10.1016/S1542-3565(05)00017-0]
44 **Marchesini G**, Bianchi G, Merli M, Amodio P, Panella C, Loguercio C, Rossi Fanelli F, Abbiati R. Nutritional supplementation with branched-chain amino acids in advanced cirrhosis: a double-blind, randomized trial. *Gastroenterology* 2003; **124**: 1792-1801 [PMID: 12806613 DOI: 10.1016/S0016-5085(03)00323-8]
45 **Sato S**, Watanabe A, Muto Y, Suzuki K, Kato A, Moriwaki H, Kato M, Nakamura T. Clinical comparison of branched-chain amino acid (l-Leucine, l-Isoleucine, l-Valine) granules and oral nutrition for hepatic insufficiency in patients with decompensated liver cirrhosis (LIV-EN study). *Hepatol Res* 2005; **31**: 232-240 [PMID: 15792640 DOI: 10.1016/j.hepres.2005.01.009]
46 **Habu D**, Nishiguchi S, Nakatani S, Lee C, Enomoto M, Tamori A, Takeda T, Ohfuji S, Fukushima W, Tanaka T, Kawamura E, Shiomi S. Comparison of the effect of BCAA granules on between decompensated and compensated cirrhosis. *Hepatogastroenterology* 2009; **56**: 1719-1723 [PMID: 20214224]
47 **Koreeda C**, Seki T, Okazaki K, Ha-Kawa SK, Sawada S. Effects of late evening snack including branched-chain amino acid on the function of hepatic parenchymal cells in patients with liver cirrhosis. *Hepatol Res* 2011; **41**: 417-422 [PMID: 21518402 DOI: 10.1111/j.1872-034X.2011.00795.x]
48 **Habu D**, Nishiguchi S, Nakatani S, Kawamura E, Lee C, Enomoto M, Tamori A, Takeda T, Tanaka T, Shiomi S. Effect of oral supplementation with branched-chain amino acid granules on serum albumin level in the early stage of cirrhosis: a randomized pilot trial. *Hepatol Res* 2003; **25**: 312-318 [PMID: 12697253 DOI: 10.1016/S1386-6346(02)00267-X]
49 **Nishiguchi S**, Habu D. Effect of oral supplementation with branched-chain amino acid granules in the early stage of cirrhosis. *Hepatol Res* 2004; **30S**: 36-41 [PMID: 15607137 DOI: 10.1016/j.hepres.2004.08.009]
50 **Chin SE**, Shepherd RW, Thomas BJ, Cleghorn GJ, Patrick MK, Wilcox JA, Ong TH, Lynch SV, Strong R. Nutritional support in children with end-stage liver disease: a randomized crossover trial of a branched-chain amino acid supplement. *Am J Clin Nutr* 1992; **56**: 158-163 [PMID: 1609753]
51 **Nakaya Y**, Okita K, Suzuki K, Moriwaki H, Kato A, Miwa Y, Shiraishi K, Okuda H, Onji M, Kanazawa H, Tsubouchi H, Kato S, Kaito M, Watanabe A, Habu D, Ito S, Ishikawa T, Kawamura N, Arakawa Y. BCAA-enriched snack improves nutritional state of cirrhosis. *Nutrition* 2007; **23**: 113-120 [PMID: 17234504 DOI: 10.1016/j.nut.2006.10.008]
52 **Ichikawa T**, Naota T, Miyaaki H, Miuma S, Isomoto H, Takeshima F, Nakao K. Effect of an oral branched chain amino acid-enriched snack in cirrhotic patients with sleep disturbance. *Hepatol Res* 2010; **40**: 971-978 [PMID: 20887332 DOI: 10.1111/j.1872-034X.2010.00701.x]
53 **Nakaya Y**, Harada N, Kakui S, Okada K, Takahashi A, Inoi J, Ito S. Severe catabolic state after prolonged fasting in cirrhotic patients: effect of oral branched-chain amino-acid-enriched nutrient mixture. *J Gastroenterol* 2002; **37**: 531-536 [PMID: 12162411 DOI: 10.1007/s005350200082]
54 **Tsuchiya M**, Sakaida I, Okamoto M, Okita K. The effect of a late evening snack in patients with liver cirrhosis. *Hepatol Res* 2005; **31**: 95-103 [PMID: 15716064 DOI: 10.1016/j.hepres.2004.11.009]
55 **Kato M**, Miwa Y, Tajika M, Hiraoka T, Muto Y, Moriwaki H. Preferential use of branched-chain amino acids as an energy substrate in patients with liver cirrhosis. *Intern Med* 1998; **37**: 429-434 [PMID: 9652895 DOI: 10.2169/internalmedicine.37.429]
56 **Yamauchi M**, Takeda K, Sakamoto K, Ohata M, Toda G. Effect of oral branched chain amino acid supplementation in the late evening on the nutritional state of patients with liver cirrhosis. *Hepatol Res* 2001; **21**: 199-204 [PMID: 11673104 DOI: 10.1016/S1386-6346(01)00105-X]
57 **Plauth M**, Cabré E, Riggio O, Assis-Camilo M, Pirlich M, Kondrup J, Ferenci P, Holm E, Vom Dahl S, Müller MJ, Nolte W. ESPEN Guidelines on Enteral Nutrition: Liver disease. *Clin Nutr* 2006; **25**: 285-294 [PMID: 16707194 DOI: 10.1016/j.clnu.2006.01.018]
58 **Kumada H**, Okanoue T, Onji M, Moriwaki H, Izumi N, Tanaka E, Chayama K, Sakisaka S, Takehara T, Oketani M, Suzuki F, Toyota J, Nomura H, Yoshioka K, Seike M, Yotsuyanagi H, Ueno Y. Guidelines for the treatment of chronic hepatitis and cirrhosis due to hepatitis C virus infection for the fiscal year 2008 in Japan. *Hepatol Res* 2010; **40**: 8-13 [PMID: 20156296 DOI: 10.1111/j.1872-034X.2009.00634.x]
59 **Bak LK**, Iversen P, Sørensen M, Keiding S, Vilstrup H, Ott P, Waagepetersen HS, Schousboe A. Metabolic fate of isoleucine in a rat model of hepatic encephalopathy and in cultured neural cells exposed to ammonia. *Metab Brain Dis* 2009; **24**: 135-145 [PMID: 19067142 DOI: 10.1007/s11011-008-9123-4]
60 **Nishikawa Y**, Ukida M, Matsuo R, Morimoto Y, Omori N, Mikami M, Tsuji T. Administration of a branched-chain amino acid preparation during hepatic failure: a study emphasizing ammonia metabolism. *Acta Med Okayama* 1994; **48**: 25-30 [PMID: 8191913]
61 **Plauth M**, Schütz T. Branched-chain amino acids in liver disease: new aspects of long known phenomena. *Curr Opin Clin Nutr Metab Care* 2011; **14**: 61-66 [PMID: 21088568 DOI: 10.1097/MCO.0b013e3283413726]
62 **Als-Nielsen B**, Koretz RL, Kjaergard LL, Gluud C. Branched-chain amino acids for hepatic encephalopathy. *Cochrane Database Syst Rev* 2003; (**2**): CD001939 [PMID: 12804416]
63 **Kanematsu T**, Koyanagi N, Matsumata T, Kitano S, Takenaka K, Sugimachi K. Lack of preventive effect of branched-chain amino acid solution on postoperative hepatic encephalopathy in patients with cirrhosis: a randomized, prospective trial. *Surgery* 1988; **104**: 482-488 [PMID: 2842882]
64 **Les I**, Doval E, García-Martínez R, Planas M, Cárdenas G, Gómez P, Flavià M, Jacas C, Mínguez B, Vergara M, Soriano G, Vila C, Esteban R, Córdoba J. Effects of branched-chain amino acids supplementation in patients with cirrhosis and a previous episode of hepatic encephalopathy: a randomized study. *Am J Gastroenterol* 2011; **106**: 1081-1088 [PMID: 21326220]
65 **Gluud LL**, Dam G, Borre M, Les I, Cordoba J, Marchesini G, Aagaard NK, Vilstrup H. Lactulose, rifaximin or branched chain amino acids for hepatic encephalopathy: what is the evidence? *Metab Brain Dis* 2013; **28**: 221-225 [PMID: 23275147 DOI: 10.1007/s11011-012-9372-0]
66 **Gluud LL**, Dam G, Borre M, Les I, Cordoba J, Marchesini G, Aagaard NK, Risum N, Vilstrup H. Oral branched-chain amino acids have a beneficial effect on manifestations of hepatic encephalopathy in a systematic review with meta-analyses of randomized controlled trials. *J Nutr* 2013; **143**: 1263-1268 [PMID: 23739310]
67 **Choudry HA**, Pan M, Karinch AM, Souba WW. Branched-chain amino acid-enriched nutritional support in surgical and cancer patients. *J Nutr* 2006; **136**: 314S-318S [PMID: 16365105]
68 **Muto Y**, Sato S, Watanabe A, Moriwaki H, Suzuki K, Kato A, Kato M, Nakamura T, Higuchi K, Nishiguchi S, Kumada H, Ohashi Y. Overweight and obesity increase the risk for liver cancer in patients with liver cirrhosis and long-term oral supplementation with branched-chain amino acid granules inhibits liver carcinogenesis in heavier patients with liver cirrhosis. *Hepatol Res* 2006; **35**: 204-214 [PMID: 16737844]
69 **Kobayashi M**, Ikeda K, Arase Y, Suzuki Y, Suzuki F, Akuta N, Hosaka T, Murashima N, Saitoh S, Someya T, Tsubota A, Kumada H. Inhibitory effect of branched-chain amino acid granules on progression of compensated liver cirrhosis due to hepatitis C virus. *J Gastroenterol* 2008; **43**: 63-70 [PMID:

18297438]
70 **Hayaishi S**, Chung H, Kudo M, Ishikawa E, Takita M, Ueda T, Kitai S, Inoue T, Yada N, Hagiwara S, Minami Y, Ueshima K. Oral branched-chain amino acid granules reduce the incidence of hepatocellular carcinoma and improve event-free survival in patients with liver cirrhosis. *Dig Dis* 2011; **29**: 326-332 [PMID: 21829025]
71 **Yoshiji H**, Noguchi R, Ikenaka Y, Kaji K, Aihara Y, Yamazaki M, Yamao J, Toyohara M, Mitoro A, Sawai M, Yoshida M, Morioka C, Fujimoto M, Uemura M, Fukui H. Combination of branched-chain amino acids and angiotensin-converting enzyme inhibitor suppresses the cumulative recurrence of hepatocellular carcinoma: a randomized control trial. *Oncol Rep* 2011; **26**: 1547-1553 [PMID: 21874260 DOI: 10.3892/or.2011.1433]
72 **Fan ST**, Lo CM, Lai EC, Chu KM, Liu CL, Wong J. Perioperative nutritional support in patients undergoing hepatectomy for hepatocellular carcinoma. *N Engl J Med* 1994; **331**: 1547-1552 [PMID: 7969324]
73 **Richter B**, Schmandra TC, Golling M, Bechstein WO. Nutritional support after open liver resection: a systematic review. *Dig Surg* 2006; **23**: 139-145 [PMID: 16809912]
74 **Togo S**, Tanaka K, Morioka D, Sugita M, Ueda M, Miura Y, Kubota T, Nagano Y, Matsuo K, Endo I, Sekido H, Shimada H. Usefulness of granular BCAA after hepatectomy for liver cancer complicated with liver cirrhosis. *Nutrition* 2005; **21**: 480-486 [PMID: 15811769]
75 **Ichikawa K**, Okabayashi T, Maeda H, Namikawa T, Iiyama T, Sugimoto T, Kobayashi M, Mimura T, Hanazaki K. Oral supplementation of branched-chain amino acids reduces early recurrence after hepatic resection in patients with hepatocellular carcinoma: a prospective study. *Surg Today* 2013; **43**: 720-726 [PMID: 22890582 DOI: 10.1007/s00595-012-0288-4]
76 **Meng WC**, Leung KL, Ho RL, Leung TW, Lau WY. Prospective randomized control study on the effect of branched-chain amino acids in patients with liver resection for hepatocellular carcinoma. *Aust N Z J Surg* 1999; **69**: 811-815 [PMID: 10553972]
77 **Okabayashi T**, Iyoki M, Sugimoto T, Kobayashi M, Hanazaki K. Oral supplementation with carbohydrate- and branched-chain amino acid-enriched nutrients improves postoperative quality of life in patients undergoing hepatic resection. *Amino Acids* 2011; **40**: 1213-1220 [PMID: 20852905 DOI: 10.1007/s00726-010-0748-3]
78 **Kuroda H**, Ushio A, Miyamoto Y, Sawara K, Oikawa K, Kasai K, Endo R, Takikawa Y, Kato A, Suzuki K. Effects of branched-chain amino acid-enriched nutrient for patients with hepatocellular carcinoma following radiofrequency ablation: a one-year prospective trial. *J Gastroenterol Hepatol* 2010; **25**: 1550-1555 [PMID: 20796154]
79 **Morihara D**, Iwata K, Hanano T, Kunimoto H, Kuno S, Fukunaga A, Yotsumoto K, Takata K, Tanaka T, Sakurai K, Iwashita H, Ueda S, Hirano G, Yokoyama K, Nakane H, Nishizawa S, Yoshikane M, Anan A, Takeyama Y, Kakumitsu S, Kitamura Y, Sakamoto M, Irie M, Shakado S, Sohda T, Watanabe H, Sakisaka S. Late-evening snack with branched-chain amino acids improves liver function after radiofrequency ablation for hepatocellular carcinoma. *Hepatol Res* 2012; **42**: 658-667 [PMID: 22380706 DOI: 10.1111/j.1872-034X.2012.00969.x]
80 **Nishikawa H**, Osaki Y, Iguchi E, Koshikawa Y, Ako S, Inuzuka T, Takeda H, Nakajima J, Matsuda F, Sakamoto A, Henmi S, Hatamaru K, Ishikawa T, Saito S, Nasu A, Kita R, Kimura T. The effect of long-term supplementation with branched-chain amino acid granules in patients with hepatitis C virus-related hepatocellular carcinoma after radiofrequency thermal ablation. *J Clin Gastroenterol* 2013; **47**: 359-366 [PMID: 23090049 DOI: 10.1097/MCG.0b013e31826be9ad]
81 **Poon RT**, Yu WC, Fan ST, Wong J. Long-term oral branched chain amino acids in patients undergoing chemoembolization for hepatocellular carcinoma: a randomized trial. *Aliment Pharmacol Ther* 2004; **19**: 779-788 [PMID: 15043519]
82 **Takeshita S**, Ichikawa T, Nakao K, Miyaaki H, Shibata H, Matsuzaki T, Muraoka T, Honda T, Otani M, Akiyama M, Miuma S, Ozawa E, Fujimito M, Eguchi K. A snack enriched with oral branched-chain amino acids prevents a fall in albumin in patients with liver cirrhosis undergoing chemoembolization for hepatocellular carcinoma. *Nutr Res* 2009; **29**: 89-93 [PMID: 19285598 DOI: 10.1016/j.nutres.2008.12.005]
83 **Nishikawa H**, Osaki Y, Inuzuka T, Takeda H, Nakajima J, Matsuda F, Henmi S, Sakamoto A, Ishikawa T, Saito S, Kita R, Kimura T. Branched-chain amino acid treatment before transcatheter arterial chemoembolization for hepatocellular carcinoma. *World J Gastroenterol* 2012; **18**: 1379-1384 [PMID: 22493552 DOI: 10.3748/wjg.v18.i12.1379]
84 **Lee IJ**, Seong J, Bae JI, You SH, Rhee Y, Lee JH. Effect of Oral Supplementation with Branched-chain Amino Acid (BCAA) during Radiotherapy in Patients with Hepatocellular Carcinoma: A Double-Blind Randomized Study. *Cancer Res Treat* 2011; **43**: 24-31 [PMID: 21509160 DOI: 10.4143/crt.2011.43.1.24]
85 **Charlton M**. Branched-chain amino acid enriched supplements as therapy for liver disease. *J Nutr* 2006; **136**: 295S-298S [PMID: 16365102]
86 **Kitagawa T**, Yokoyama Y, Kokuryo T, Nagino M. Protective effects of branched-chain amino acids on hepatic ischemia-reperfusion-induced liver injury in rats: a direct attenuation of Kupffer cell activation. *Am J Physiol Gastrointest Liver Physiol* 2013; **304**: G346-G355 [PMID: 23275614 DOI: 10.1152/ajpgi.00391.2012]
87 **Deshpande G**, Adachi N, Liu K, Motoki A, Mitsuyo T, Nagaro T, Arai T. Recovery of brain dopamine metabolism by branched-chain amino acids in rats with acute hepatic failure. *J Neurosurg Anesthesiol* 2007; **19**: 243-248 [PMID: 17893576]
88 **Kuwahata M**, Kuramoto Y, Tomoe Y, Sugata E, Segawa H, Ito M, Oka T, Miyamoto K. Posttranscriptional regulation of albumin gene expression by branched-chain amino acids in rats with acute liver injury. *Biochim Biophys Acta* 2004; **1739**: 62-69 [PMID: 15607118]
89 **Record CO**, Buxton B, Chase RA, Curzon G, Murray-Lyon IM, Williams R. Plasma and brain amino acids in fulminant hepatic failure and their relationship to hepatic encephalopathy. *Eur J Clin Invest* 1976; **6**: 387-394 [PMID: 10164]
90 **Rosen HM**, Yoshimura N, Hodgman JM, Fischer JE. Plasma amino acid patterns in hepatic encephalopathy of differing etiology. *Gastroenterology* 1977; **72**: 483-487 [PMID: 832796]
91 **O'Shea RS**, McCullough AJ. Treatment of alcoholic hepatitis. *Clin Liver Dis* 2005; **9**: 103-134 [PMID: 15763232]
92 **Arrese M**, Riquelme A, Soza A. Insulin resistance, hepatic steatosis and hepatitis C: a complex relationship with relevant clinical implications. *Ann Hepatol* 2010; **9** Suppl: 112-118 [PMID: 20714007]
93 **Kawaguchi T**, Yamagishi S, Sata M. Branched-chain amino acids and pigment epithelium-derived factor: novel therapeutic agents for hepatitis c virus-associated insulin resistance. *Curr Med Chem* 2009; **16**: 4843-4857 [PMID: 19929783]
94 **Takeshita Y**, Takamura T, Kita Y, Ando H, Ueda T, Kato K, Misu H, Sunagozaka H, Sakai Y, Yamashita T, Mizukoshi E, Honda M, Kaneko S. Beneficial effect of branched-chain amino acid supplementation on glycemic control in chronic hepatitis C patients with insulin resistance: implications for type 2 diabetes. *Metabolism* 2012; **61**: 1388-1394 [PMID: 22520843 DOI: 10.1016/j.metabol.2012.03.011]
95 **Honda M**, Takehana K, Sakai A, Tagata Y, Shirasaki T, Nishitani S, Muramatsu T, Yamashita T, Nakamoto Y, Mizukoshi E, Sakai Y, Yamashita T, Nakamura M, Shimakami T, Yi M, Lemon SM, Suzuki T, Wakita T, Kaneko S. Malnutrition impairs interferon signaling through mTOR and FoxO pathways in patients with chronic hepatitis C. *Gastroen-*

terology 2011; **141**: 128-140, 140.e1-2 [PMID: 21458454 DOI: 10.1053/j.gastro.2011.03.051]
96 **Kawaguchi T**, Torimura T, Takata A, Satomi S, Sata M. Valine, a branched-chain amino Acid, reduced HCV viral load and led to eradication of HCV by interferon therapy in a decompensated cirrhotic patient. *Case Rep Gastroenterol* 2012; **6**: 660-667 [PMID: 23185147 DOI: 10.1159/000343094]
97 **Nagao Y**, Kawaguchi T, Ide T, Sata M. Effect of branched-chain amino acid-enriched nutritional supplementation on interferon therapy in Japanese patients with chronic hepatitis C virus infection: a retrospective study. *Virol J* 2012; **9**: 282 [PMID: 23173649 DOI: 10.1186/1743-422X-9-282]
98 **Kaido T**, Mori A, Oike F, Mizumoto M, Ogura Y, Hata K, Yoshizawa A, Iida T, Uemoto S. Impact of pretransplant nutritional status in patients undergoing liver transplantation. *Hepatogastroenterology* 2010; **57**: 1489-1492 [PMID: 21443108]
99 **Kawamura E**, Habu D, Morikawa H, Enomoto M, Kawabe J, Tamori A, Sakaguchi H, Saeki S, Kawada N, Shiomi S. A randomized pilot trial of oral branched-chain amino acids in early cirrhosis: validation using prognostic markers for pre-liver transplant status. *Liver Transpl* 2009; **15**: 790-797 [PMID: 19562716 DOI: 10.1002/lt.21758]
100 **Shirabe K**, Yoshimatsu M, Motomura T, Takeishi K, Toshima T, Muto J, Matono R, Taketomi A, Uchiyama H, Maehara Y. Beneficial effects of supplementation with branched-chain amino acids on postoperative bacteremia in living donor liver transplant recipients. *Liver Transpl* 2011; **17**: 1073-1080 [PMID: 21542128 DOI: 10.1002/lt.22324]
101 **Kawaguchi T**, Izumi N, Charlton MR, Sata M. Branched-chain amino acids as pharmacological nutrients in chronic liver disease. *Hepatology* 2011; **54**: 1063-1070 [PMID: 21563202]
102 **Tabaru A**, Shirohara H, Moriyama A, Otsuki M. Effects of branched-chain-enriched amino acid solution on insulin and glucagon secretion and blood glucose level in liver cirrhosis. *Scand J Gastroenterol* 1998; **33**: 853-859 [PMID: 9754734]
103 **Korenaga K**, Korenaga M, Uchida K, Yamasaki T, Sakaida I. Effects of a late evening snack combined with alpha-glucosidase inhibitor on liver cirrhosis. *Hepatol Res* 2008; **38**: 1087-1097 [PMID: 18647188]
104 **Sakaida I**, Tsuchiya M, Okamoto M, Okita K. Late evening snack and the change of blood glucose level in patients with liver cirrhosis. *Hepatol Res* 2004; **30S**: 67-72 [PMID: 15607142]
105 **Kawaguchi T**, Taniguchi E, Itou M, Sumie S, Oriishi T, Matsuoka H, Nagao Y, Sata M. Branched-chain amino acids improve insulin resistance in patients with hepatitis C virus-related liver disease: report of two cases. *Liver Int* 2007; **27**: 1287-1292 [PMID: 17919242]
106 **Miyake T**, Abe M, Furukawa S, Tokumoto Y, Toshimitsu K, Ueda T, Yamamoto S, Hirooka M, Kumagi T, Hiasa Y, Matsuura B, Onji M. Long-term branched-chain amino acid supplementation improves glucose tolerance in patients with nonalcoholic steatohepatitis-related cirrhosis. *Intern Med* 2012; **51**: 2151-2155 [PMID: 22892494]

P- Reviewers: Faintuch J, Loguercio C, Ramsay M
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.v19.i43.7630

World J Gastroenterol 2013 November 21; 19(43): 7630-7638
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Liver diseases in pregnancy: Diseases not unique to pregnancy

Ashraf A Almashhrawi, Khulood T Ahmed, Rubayat N Rahman, Ghassan M Hammoud, Jamal A Ibdah

Ashraf A Almashhrawi, Khulood T Ahmed, Rubayat N Rahman, Ghassan M Hammoud, Jamal A Ibdah, Division of Gastroenterology and Hepatology, University of Missouri-Columbia, Columbia, MO 65212, United States

Author contributions: Almashhrawi AA wrote and revised the manuscript; Ahmed KT, Rahman RN, and Hammoud GM were involved in reviewing the literature and collecting data; and Ibdah JA conceived the topic, contributed to the writing, analyzed and edited the manuscript, and provided overall intellectual input into the design and execution of the manuscript.

Correspondence to: Jamal A Ibdah, MD, PhD, Professor, Director, Division of Gastroenterology and Hepatology, University of Missouri-Columbia, 319 jesse hall, Columbia, MO 65212, United States. ibdahj@health.missouri.edu

Telephone: +1-573-8827349 Fax: +1-573-8844595
Received: June 10, 2013 Revised: August 5, 2013
Accepted: September 4, 2013
Published online: November 21, 2013

Abstract

Pregnancy is a special clinical state with several normal physiological changes that influence body organs including the liver. Liver disease can cause significant morbidity and mortality in both pregnant women and their infants. Few challenges arise in reaching an accurate diagnosis in light of such physiological changes. Laboratory test results should be carefully interpreted and the knowledge of what normal changes to expect is prudent to avoid clinical misjudgment. Other challenges entail the methods of treatment and their safety for both the mother and the baby. This review summarizes liver diseases that are not unique to pregnancy. We focus on viral hepatitis and its mode of transmission, diagnosis, effect on the pregnancy, the mother, the infant, treatment, and breast-feeding. Autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, Wilson's disease, Budd Chiari and portal vein thrombosis in pregnancy are also discussed. Pregnancy is rare in patients with cirrhosis because of the metabolic and hormonal changes associated with cirrhosis. Variceal bleeding can happen in up to 38% of cirrhotic pregnant women. Management of portal hypertension during pregnancy is discussed. Pregnancy increases the pathogenicity leading to an increase in the rate of gallstones. We discuss some of the interventions for gallstones in pregnancy if symptoms arise. Finally, we provide an overview of some of the options in managing hepatic adenomas and hepatocellular carcinoma during pregnancy.

Key words: Liver; Pregnancy; Viral hepatitis; Autoimmune; Cirrhosis; Gallstones; Adenoma

Core tip: Pregnancy is a special clinical state with several normal physiological changes that influence body organs including the liver. Liver disease can cause significant morbidity and mortality in both pregnant women and their infants. Challenges involve making the diagnosis and the methods of treatment and their safety for both the mother and the baby. This review summarizes liver diseases that are not unique to pregnancy.

Almashhrawi AA, Ahmed KT, Rahman RN, Hammoud GM, Ibdah JA. Liver diseases in pregnancy: Diseases not unique to pregnancy. *World J Gastroenterol* 2013; 19(43): 7630-7638 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7630.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7630

INTRODUCTION

Although not unique to pregnancy, liver diseases reviewed here can have significant consequences on pregnant women and their infants.

Approach to the diagnosis of liver conditions in preg-

Table 1 Normal physiological alterations in liver tests in pregnancy

Test	First trimester	Second/third trimesters
Albumin	↓	↓
ALT	N	N
AST	N	N
Total bilirubin	↓	↓
Alkaline phosphatase	N	↑
GGT	N	↓
5′-nucleotidase	N	May increase in second and third trimesters
Fasting total bile acids	N	N
Prothrombin time	N	N

N: No change; ↑: Increase; ↓: Decrease; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transpeptidase.

Table 2 Interpretation of hepatitis B blood tests

Test	HBsAg	Anti-HBs	Total Anti-HBc	Anti-HBc IgM	HBV DNA
Acute infection	+	-	+	+	+
Resolved infection with natural immunity	-	+	+	-	-
Immunity through vaccination	-	+	-	-	-
Chronic infection	+	-	+	-	+/-
Different possibilities[1]	-	-	+	-	-

[1]Could represent resolving acute infection, resolved infection (most likely), chronic infection with low viral load or false positive. HBsAg: Hepatitis B surface antigen; Anti-HBs: Hepatitis B surface antibody; Total anti-HBc: Total hepatitis B core antibody; Anti-HBc IgM: Hepatitis B core antibody immunoglobulin M; HBV: Hepatitis B virus.

nant women should take into consideration the physiological changes during pregnancy that allow for normal fetal development. Sex hormones such as estrogen and progesterone increase progressively during pregnancy. This increase has an influence on hepatic metabolic, synthetic, and excretory functions[1]. During late pregnancy, biliary excretion of few compounds can be reduced. Furthermore, reduction in serum protein concentrations secondary to reversible hemodilution resulting from expanding plasma volume while pregnant is reflected by alterations in some liver function tests (Table 1).

Whereas nausea and vomiting are common in early pregnancy, those should not be considered normal in the second or third trimesters and ought to be investigated[2]. Jaundice and generalized pruritus are not normal features in pregnancy. Spider nevi and palmar erythema were found up to 66% and 63% respectively by the end of normal pregnancy in one study[3]. Most of those were reversible after delivery.

Unique aspects such as the effect of the disease on pregnancy, the effect of the pregnancy on disease progression, the use of specific therapies during pregnancy, and issues related to breast-feeding are discussed.

VIRAL HEPATITIS AND PREGNANCY

Hepatitis A virus

Hepatitis A virus (HAV) is an RNA virus that transmits through fecal-oral route, usually through contaminated water or food. Overall incidence is 9.1 per 100000 in the United States and less than 1:1000 pregnancies. Clinical presentation ensues within 2-4 wk of exposure. Generally, HAV does not result in chronic infection. Acute hepatitis A starts with prodromal symptoms including anorexia, malaise, nausea and vomiting, and progresses into jaundice and elevated liver transaminases. Presence of HAV immunoglobulin M (IgM) antibodies confirms the acute infection. Management is supportive care including optimizing hydration and nutrition. Rarely acute hepatitis A can lead to fulminant hepatic failure. Inactivated HAV vaccine and immunoglobulin prophylaxis are safe in pregnancy[4]. Although vertical transmission has been reported, intrauterine transmission is rare[5-7]. Fecal-oral transmission during birth is possible. No cases of teratogenicity were reported, but maternal complications such as preterm labor were described. Susceptible woman should receive vaccination. Breast-feeding is not contraindicated in acute hepatitis A with following appropriate hygiene measures.

Hepatitis B virus

Hepatitis B virus (HBV) is a DNA virus that is highly infectious and transmits through intravenous route, sexual contact, and vertically from the mother to her fetus. It can present both as an acute or chronic infection. Pregnancy does not affect the course of infection directly. Fortunately, since universal children vaccination for hepatitis B was implemented in 1992, the numbers of vertically transmitted chronic hepatitis B cases, and its complications such as hepatocellular carcinoma have dropped[8-10]. Prenatal screening for HBV is standard of care in many countries including the United States. Those susceptible should be vaccinated. Pregnant women exposed to HBV should receive HBV immunoglobulins (HBIG) within 72 h of exposure in addition to the vaccination series. Infants with infected mothers should receive both immunoglobulins and vaccination series at the time of delivery. While acute infection can present with a viral syndrome and jaundice such as that of acute hepatitis A infection, chronic infection is usually asymptomatic and diagnosis can be made relying on serum serology testing. A summary of the tests used in hepatitis B diagnosis and their interpretation is displayed in Table 2.

Treatment should follow guidelines published by medical societies such as the American Association for the Study of Liver Disease (AASLD)[11], the European Association for the Study of the Liver[12], or the Asian Pacific Association for the Study of the Liver[13]. In the United States, we recommend referring infected pregnant women to the state's perinatal hepatitis B prevention program[14], that is CDC-funded (centers for disease control and prevention), and to liver specialists for optimizing counseling and treatment.

There are seven Food and Drug Administration (FDA)-approved medications for the treatment of hepati-

Table 3 Food and Drug Administration approved medications for hepatitis B treatment

Generic name	Trade name	Company	Approved for HBV treatment
Interferons			
Interferon α-2b, recombinant	Intron® A	Schering Corporation/ Merck and Co	1992
Perinterferon α-2a	Pegasys®	Genentech/Roche group	2005
Nucleosides/nucleotides			
Lamivudine[1]	EPIVIR-HBV®	GlaxoSmithKline	1998
Adefovir dipivoxil	HEPSERA™	Gilead Sciences	2002
Entecavir	BARACLUDE™	Bristol-Myers Squibb	2005
Telbivudine[2]	TYZEKA™	Novartis	2006
Tenofovir[2]	Viread	Gilead Sciences	2008

[1]Pregnancy risk category C, can be used in the third trimester; [2]Pregnancy risk category B. HBV: Hepatitis B virus.

tis B (Table 3) in non-pregnant patients. Interferon use is contraindicated in pregnancy. Tenofovir and Telbivudine belong to pregnancy risk category B; all others belong to category C. The choice to treat or not should be weighed in light of benefits versus risks for both the mother and her fetus. Those with higher viral load (serum HBV DNA > 10^8 copies/mL) were at higher risk for vertical transmission in one study[15]. Wen *et al*[16] showed recently that the adjusted odds ratio of transmission for each log10 copy/mL increase, is 3.49 ($P = 0.001$), with predictive rates of infection at maternal viral load levels of 7, 8, and 9-log10 copies/ml of 6.6% ($P = 0.033$), 14.6% ($P = 0.001$), and 27.7% ($P < 0.001$), respectively. Therefore, it is reasonable to treat those women or women with previous infected children, especially towards the end of pregnancy (from week 28 and up), with risk category B drugs or Lamivudine (increases birth defects if used in 1st trimester)[17,18]. In a meta-analysis, significant drop in the risk of vertical transmission was found in those who succeeded to lower HBV DNA below 10^6 copies/mL[18]. Telbivudine was used safely and with good efficacy in reducing transmission (0% *vs* 8%; $P = 0.002$) in a recent study[19].

Although cesarean section is proposed as a measure to lower the risk of transmission, particularly in women with high viral loads towards term, there is a conflicting evidence regarding choosing cesarean section versus vaginal delivery to lower the risk of vertical transmission[20,21]. Breast-feeding should be encouraged for infants receiving HBIG and vaccination[22-25]. On the other hand, no adequate evidence of the safety of breast-feeding in mothers receiving antiviral therapy is available and women on antiviral therapy with lamivudine, telbivudine or tenofovir should be discouraged from breast-feeding[26-28].

Hepatitis C virus

With prevalence around 1.6%, chronic hepatitis C infection continues to present a big public health concern in the United States. The majority of those patients, left untreated, will progress to cirrhosis with expected peak in prevalence around the year 2030, with expected medical cost exceeding $85 billion[29]. Generally, all high-risk patients should be screened for hepatitis C virus (HCV) following CDC and AASLD guidelines. Those include children born to HCV infected mothers. While there is no approved medicine to treat chronic hepatitis C in pregnant women, those should be referred to liver experts for education regarding options of treatment after delivery and preventive measures to slow the progression of the disease. HCV antibodies ELISA testing is a sensitive test and carries high positive predictive value in high-risk patients. Diagnosis can be confirmed using HCV RNA polymerase chain reaction (PCR). There are several therapies for hepatitis C that are under investigation currently. Some of those could prove safe to use in pregnancy in the future. Pregnant women with hepatitis C should be educated about the mode of transmission and how to reduce the risk, smoking cessation, alcohol abstinence, and vaccination for hepatitis A and hepatitis B. They should also be screened for hepatitis B and human immunodeficiency virus (HIV) infection. Women undergoing treatment for hepatitis C, or those with partners undergoing treatment for hepatitis C, should avoid pregnancy by using at least 2 forms of barrier contraception, for the period of treatment and 6 mo after.

Infants of hepatitis C infected- mothers were at higher risk for low birth weight, being small for gestational age, or requiring intensive care upon birth in one report[30]. The risk of vertical transmission is approximately 4%. This risk increased up to 19.4% when co-infected with HIV[31-35]. High viral load also increase the risk for vertical transmission. HCV transmission could occur through viral trancytosis across trophoblast cells mediated by HCV receptors expressed on trophoblasts or through some form of injury that influences the placental barrier[36]. Although there were few reports of increased risk of transmission with premature rupture of membrane, more than 6 h before delivery, mode of delivery was not found to change the risk of hepatitis C transmission[31,37-39]. As the new era of direct antiviral agents is evolving, treating hepatitis C during pregnancy may become an option and thus the possibility of reducing the risk of transmission[40].

Breast-feeding is considered safe when nipples are not cracked or bleeding according to CDC recommendations.

Hepatitis D virus

Hepatitis D virus (HDV) is an RNA virus that requires

hepatitis B surface antigen for replication. Anti-HDV antibodies establish the diagnosis. Although vertical transmission is possible, hepatitis D is preventable by preventing HBV transmission[41].

Hepatitis E virus

Hepatitis E virus (HEV) is an RNA virus that is usually transmitted through fecal-oral means, although transmission via infected blood products and vertical transmission has been reported[42]. It is usually a self-limiting disease in immunocompetent patients. Hepatitis E can cause significant disease in patients with chronic liver disease and can present in a chronic form leading to fibrosis in immunocompromised individuals[43]. Pregnant women in highly endemic areas are particularly at risk with up to 60% developing fulminant hepatic failure with a maternal death rate of up to 31%[44,45]. A review from Bangladesh suggests it is responsible for 9.8% of pregnancy-related deaths[46]. On the other hand, the severity of the disease was not different between pregnant and non-pregnant women in non-endemic places such as the United States and Europe. A report suggested that such variance in severity between endemic and non-endemic areas might be related to different genotypes of HEV[47]. Other studies suggested that pregnancy per se is not a poor prognostic factor for those who developed acute liver failure[48]. To a lesser extent, hepatitis E is prevalent in some western countries, particularly genotype 3.

Vertical transmission was described up to 78.9% with infant mortality of 40%[42]. The level of viremia appears to be associated with the severity of the disease during pregnancy[49]. Despite such high mortality, current treatment remains supportive. Pregnant woman seeking travel to endemic areas should be counseled about the risk of hepatitis E, and be advised to avoid unpurified water, uncooked fruit, vegetables, and shellfish.

Hepatitis E vaccines have been developed and evaluated in trials but has not been approved for commercial use yet. Their utility is yet to be determined[50-54].

Herpes simplex virus

32 out of 137 cases of herpes simplex virus (HSV) hepatitis were pregnant women in one report, suggesting their susceptibility[55]. Although rare, HSV hepatitis carries a very high mortality (39%) if inappropriately treated[56]. Providers should have high index of suspicion in this patient group in the appropriate clinical setting; elevated liver transaminases usually 100 times upper level of normal with typically normal or mildly elevated bilirubin (anicteric hepatitis)[57-60]. Serology testing including anti-HSV IgM should be ordered. HSV PCR can be ordered as well to confirm diagnosis. Recent study has revealed that HSV DNA load correlated with liver transaminase levels and disease severity[61]. Although no strong evidence to support starting Acyclovir in patients with indeterminate acute liver failure, clinicians should consider empirical therapy with acyclovir when HSV hepatitis is suspected[59]. Liver biopsy with appropriate immunohistochemistry staining can be useful, but usually is avoided because of its invasive nature, coagulopathy and because of the potential delay in results/treatment.

AUTOIMMUNE HEPATITIS AND PREGNANCY

Autoimmune hepatitis is a disease characterized by elevated liver aminotransferases, hypergammaglobulinemia, and positive serum autoantibodies. Autoimmune hepatitis and pregnancy (AIH) is more common in females, especially those in childbearing ages. It can happen during pregnancy and may not follow consistent pattern. Normalization of liver aminotransferases has been described in patients with no treatment[62]. This normalization could be related to the immunotolerant state that predominates pregnancy. On the other hand, flare-ups have been reported during and after pregnancy[63]. Prematurity and fetal-loss were described in those patients[64]. A link was observed between antibodies to soluble liver antigen/liver-pancreas and ribonucleoprotein/Sjögren's syndrome A and adverse outcomes[65]. Inadequate disease control in the year prior to pregnancy and the absence of treatment during pregnancy were associated with unfavorable outcomes in a recent study[66].

Although the patients should be counseled about possible adverse outcomes, pregnancy appears to be safe in well-controlled AIH women[67]. Special considerations should be given to the postpartum period as flare-ups may occur frequently, and treatment should be resumed preemptively two weeks before delivery and maintained thereafter[68]. Immunosuppressive therapy with steroids and agents such as azathioprine is the mainstay for treatment of AIH. Azathioprine use during pregnancy is generally safe (despite reports of birth defects in animal models)[64].

PRIMARY BILIARY CIRRHOSIS/PRIMARY SCLEROSING CHOLANGITIS AND PREGNANCY

There is limited data about pregnancy in patients with primary biliary cirrhosis. Reports have ranged from normal course of pregnancy and good fetal outcomes to poor prognosis for both mother and fetus[69,70]. Earlier diagnosis and the use of ursodeoxycholic acid (UDCA) in treatment, which has been used safely in pregnancy, have been linked to favorable outcomes[71]. Primary sclerosing cholangitis did not appear to reduce fertility and resulted in good outcomes, in one report. UDCA was successfully used to control pruritus in this cohort[72].

WILSON'S DISEASE AND PREGNANCY

Wilson's disease is an autosomal recessive disease with

Table 4 Options for portal hypertension management in pregnancy

Esophageal varices	Nonselective β-blockers
	Endoscopic and ligation and/or sclerotherapy
	TIPS: Data on TIPS and pregnancy is limited
Ascites	Sodium (salt) restriction, diuretics
Hepatic encephalopathy	Lactulose, rifaximin

TIPS: Transjugular portosystemic shunt.

prevalence of 1:30000 to 1:50000[73]. It affects hepatic copper transport with inhibition of biliary excretion, resulting in excess circulating copper and deposition in organs such as the liver and the brain. Cases of reduced fertility and recurrent spontaneous abortions in untreated women were reported[74]. Chelation therapy using *D*-penicillamine or trientine, or the use of zinc to reduce intestinal absorption of copper, have been the mainstay therapy for Wilson's disease. Zinc has been used with minimal teratogenicity during pregnancy[75]. Although teratogenic effects of *D*-penicillamine in humans and animals, and teratogenic effects of trientine in animals were described[76,77], therapy should not be discontinued as this can result in severe hemolysis, worsening of liver function and even death. Even though zinc dosages can be maintained during pregnancy, AASLD recommends lowering *D*-penicillamine and trientine to the minimum needed (usually 25%-50% of the pre-pregnancy dose)[78], particularly towards term to aid in wound healing. Baseline dosages can be resumed postnatal. The mother should be counseled, and both the mother and her fetus should be monitored closely during pregnancy. Breastfeeding is discouraged as *D*-pencillamine can be harmful to the infant and safety has not been established with trientene and zinc.

GALLSTONES AND PREGNANCY

Physiological changes during pregnancy particularly hormonal changes lead to decrease in contractility of the gallbladder and changes in bile content, with increase in cholesterol saturation, resulting in increase in lethogenicity of the bile[79]. Incidence of gallstones is up to 12% in pregnant women[80]. Those typically remain asymptomatic. The patient can present with biliary pain, gallstone pancreatitis, or less likely acute cholecystitis. Other manifestations such as choledocholithiasis and cholangitis can also happen. Management is mostly conservative with hydration and antibiotics if indicated. In more severe cases, cholecystectomy can be indicated. Endoscopic retrograde cholangiopancreatography (ERCP) can also be used with taking precautions to minimize radiation exposure of the fetus. In general, surgical procedures are the safest in the second trimester. ERCP was reported to be associated with higher risk for preterm pregnancy and low birthweight when performed in the first trimester. Post-ERCP pancreatitis rate was higher in pregnancy than general population[81-85].

CIRRHOSIS/ PORTAL HYPERTENSION AND PREGNANCY

Pregnancy in cirrhotic women is rare, probably because of low prevalence of cirrhosis in reproductive age group (45 in 100000) and also due to amenorrhea and anovulation, likely related to metabolic and hormonal derangements[86]. The physiological increase in plasma volume during pregnancy can worsen portal hypertension, resulting in increase risk of variceal bleeding. Variceal bleeding can happen in up to 38% of cirrhotic pregnant women. This is even higher in those with known portal hypertension. Those with known varices have a 78% chance of bleeding[87]. AASLD recommends screening for esophageal varices by the second trimester, as the risk of bleeding appears to be highest at that time. Women with cirrhosis planning to become pregnant should be screened before conception by endoscopy and prophylaxis (with nonselective beta blockers) should be started as recommended by AASLD guidelines. Complications of portal hypertension in pregnancy can be as high as 50% resulting in high mortality rate of up to 18%, and higher risk for fetal loss[88]. Pregnancy should be avoided in women with previous history of variceal bleeding and liver insufficiency. Means such as early forceps delivery or vacuum extraction should be considered to prevent excessive straining during vaginal delivery. Management options of complications of portal hypertension are summarized in Table 4. All medications used during pregnancy should be checked as of which risk category they fall under according to the FDA classification before prescribing (Tables 5 and 6).

HEPATOCELLULAR ADENOMA AND PREGNANCY

The incidence of hepatocellular adenoma has increased since the introduction of oral contraceptives. There is a link between pregnancy and liver adenomas secondary to higher levels of hormones[89]. Rupture of adenomas has resulted in maternal mortality of a 44% and fetal loss of 38% in one study[90]. Adenoma rupture risk increases towards the end of pregnancy[91]. Women with adenomas > 5 cm or those with previous complications with adenomas, should avoid subsequent pregnancies. Those pregnant with smaller adenomas should be monitored closely with serial ultrasound imaging. If the lesion is progressively enlarging, or 5 cm in size or bigger, surgical resection should be considered[90]. Radiofrequency ablation is another modality that can be used in the treatment of hepatic adenomas[91-93]. Close monitoring of the lesion should continue in the postpartum period as well.

HEPATOCELLULAR CARCINOMA AND PREGNANCY

Although rare, hepatocellular carcinoma has been reported during pregnancy. Fibrolamellar variant of hepatocel-

Table 5 The Food and Drug Administration pregnancy risk categories of medicines

Pregnancy category	Definition
A	Controlled studies show no risk
B	Animal studies show no risk, and there are no human controlled studies. Or animal studies may have revealed an adverse effect that was not reproduced in human controlled studies
C	No human studies and either animal studies show an adverse effect or there are no studies available. Use if the risk is justified
D	Positive evidence of risk in human studies, only if the potential benefits outweigh the risk
X	Contraindicated in pregnancy: Risk is confirmed in animal and human studies and outweighs any advantage

Table 6 Food and Drug Administration pregnancy risk categories of some liver disease medications

Medicine	Pregnancy category	Medicine	Pregnancy category
Nadolol	C	Ribavirin	X
Propranolol	C	Telaprevir	B
Rifaximin	C	Boceprevir	B
Lactulose	B	Tenofovir	B
Furosemide	C	Entecavir	C
Spironolactone	C	Telbuvidine	B
Corticosteroids	B	Adefovir	C
Azathioprine	D	Lamuvidine	C
Cyclosporin	C	Acyclovir	B
Mycophenolate mofetil	D	Ursodeoxycholic acid	B
Tacrolimus	C	Penicillamine	D
Sirolimus	C	Trientine	C
Antithymocyte globulin	C	Zinc sulfate	C
Pegylated interferon	C (contraindicated in pregnancy)	Interferon alpha 2b	C (contraindicated in pregnancy)

lular carcinoma (HCC) was also reported[94-96]. Pregnant women with HCC can have shorter median survival than those non-pregnant. Higher levels of estrogen and immune suppression during pregnancy can play a role with HCC progression[97]. Modalities such as surgical resection and radiofrequency ablation can be used in selected patients. Limited data are available about the management of hepatocellular carcinoma in pregnancy.

HEPATIC VEIN THROMBOSIS/PORTAL VEIN THROMBOSIS AND PREGNANCY

Budd-Chiari syndrome (BCS) is rare in pregnancy but can have grave consequences for both the mother and her fetus. The physiological hypercoagulable state can contribute in BCS development in pregnancy. Other predisposing factors are factor V Leiden mutation and prothrombin gene mutations. BCS entails thrombosis of the hepatic vein resulting in passive congestion of the hepatic sinusoids leading to ischemia and portal hypertension. Low molecular weight heparin should be started if no contraindications. Extreme measures such as portacaval shunting and liver transplantation during pregnancy were reported[98,99]. Subsequent pregnancies are not absolutely contraindicated with appropriately treated disease. The mother should be counseled about the possible maternal and fetal unfavorable outcomes.

Portal vein thrombosis (PVT) is rare and can also occur during pregnancy. Local causes such as cirrhosis, intra-abdominal infections, or malignancies may predispose to PVT. Systemic disorders resulting in hypercoagulable state such as factor V Leiden mutation, anti-phospholipid syndrome, or myeloproliferative disorders should be also excluded. In acute portal vein thrombosis, anti-coagulation should be used for 3 mo at the least. Patients with chronic portal vein thrombosis should be screened for gastroesophageal varices and should be treated accordingly[100].

CONCLUSION

Pregnant women can have a variety of liver diseases with different incidences. Clinicians should be aware of the clinical presentations and be able to manage those conditions with special attention to the peculiarities in relation to the mother and her infant. In this review we have summarized several of the liver diseases that can happen during pregnancy and offered an overview of their management.

REFERENCES

1 **Van Thiel DH**, Gavaler JS. Pregnancy-associated sex steroids and their effects on the liver. *Semin Liver Dis* 1987; **7**: 1-7 [PMID: 3589711 DOI: 10.1055/s-2008-1040558]
2 **Bacq Y**, Riely C. The liver in pregnancy. In: Schiff's diseases of the liver. 9th ed. Philadelphia: Lippincott Williams and Wilkins; 2003: 1435–1457
3 **Bean WB**, Cogswell R. Vascular changes of the skin in pregnancy; vascular spiders and palmar erythema. *Surg Gynecol Obstet* 1949; **88**: 739-752 [PMID: 18130313]
4 **Magriples U**. Hepatitis in pregnancy. *Semin Perinatol* 1998; **22**: 112-117 [PMID: 9638905]
5 **Motte A**, Blanc J, Minodier P, Colson P. Acute hepatitis A in a pregnant woman at delivery. *Int J Infect Dis* 2009; **13**: e49-e51 [PMID: 18774327 DOI: 10.1016/j.ijid.2008.06.009]
6 **Selander B**, Bläckberg J, Widell A, Johansson PJ. No evidence

of intrauterine transmission of hepatitis A virus from a mother to a premature infant. *Acta Paediatr* 2009; **98**: 1603-1606 [PMID: 19558626 DOI: 10.1111/j.1651-2227.2009.01402.x]
7 **Renge RL**, Dani VS, Chitambar SD, Arankalle VA. Vertical transmission of hepatitis A. *Indian J Pediatr* 2002; **69**: 535-536 [PMID: 12139145]
8 **Beasley RP**. Rocks along the road to the control of HBV and HCC. *Ann Epidemiol* 2009; **19**: 231-234 [PMID: 19344859 DOI: 10.1016/j.annepidem.2009.01.017]
9 **Ni YH**, Chen DS. Hepatitis B vaccination in children: the Taiwan experience. *Pathol Biol* (Paris) 2010; **58**: 296-300 [PMID: 20116181 DOI: 10.1016/j.patbio.2009.11.002]
10 **Tajiri H**, Tanaka H, Brooks S, Takano T. Reduction of hepatocellular carcinoma in childhood after introduction of selective vaccination against hepatitis B virus for infants born to HBV carrier mothers. *Cancer Causes Control* 2011; **22**: 523-527 [PMID: 21191808 DOI: 10.1007/s10552-010-9721-4]
11 **Lok AS**, McMahon BJ. Chronic hepatitis B: update 2009. *Hepatology* 2009; **50**: 661-662 [PMID: 19714720 DOI: 10.1002/hep.23190]
12 **European Association For The Study Of The Liver**. EASL clinical practice guidelines: Management of chronic hepatitis B virus infection. *J Hepatol* 2012; **57**: 167-185 [PMID: 22436845]
13 **Liaw YF**, Leung N, Guan R, Lau GK, Merican I, McCaughan G, Gane E, Kao JH, Omata M. Asian-Pacific consensus statement on the management of chronic hepatitis B: a 2005 update. *Liver Int* 2005; **25**: 472-489 [PMID: 15910483]
14 State perinatal hepatitis B prevention program (PHBPP). Available from: URL: http: //www.cdc.gov/hepatitis/Partners/PeriHepBCoord.htm
15 **Wiseman E**, Fraser MA, Holden S, Glass A, Kidson BL, Heron LG, Maley MW, Ayres A, Locarnini SA, Levy MT. Perinatal transmission of hepatitis B virus: an Australian experience. *Med J Aust* 2009; **190**: 489-492 [PMID: 19413519]
16 **Wen WH**, Chang MH, Zhao LL, Ni YH, Hsu HY, Wu JF, Chen PJ, Chen DS, Chen HL. Mother-to-infant transmission of hepatitis B virus infection: significance of maternal viral load and strategies for intervention. *J Hepatol* 2013; **59**: 24-30 [PMID: 23485519 DOI: 10.1016/j.jhep.2013.02.015]
17 **Xu WM**, Cui YT, Wang L, Yang H, Liang ZQ, Li XM, Zhang SL, Qiao FY, Campbell F, Chang CN, Gardner S, Atkins M. Lamivudine in late pregnancy to prevent perinatal transmission of hepatitis B virus infection: a multicentre, randomized, double-blind, placebo-controlled study. *J Viral Hepat* 2009; **16**: 94-103 [PMID: 19175878 DOI: 10.1111/j.1365-2893.2008.01056.x]
18 **Han L**, Zhang HW, Xie JX, Zhang Q, Wang HY, Cao GW. A meta-analysis of lamivudine for interruption of mother-to-child transmission of hepatitis B virus. *World J Gastroenterol* 2011; **17**: 4321-4333 [PMID: 22090789 DOI: 10.3748/wjg.v17.i38.4321]
19 **Han GR**, Cao MK, Zhao W, Jiang HX, Wang CM, Bai SF, Yue X, Wang GJ, Tang X, Fang ZX. A prospective and open-label study for the efficacy and safety of telbivudine in pregnancy for the prevention of perinatal transmission of hepatitis B virus infection. *J Hepatol* 2011; **55**: 1215-1221 [PMID: 21703206 DOI: 10.1016/j.jhep.2011.02.032]
20 **Wang J**, Zhu Q, Zhang X. Effect of delivery mode on maternal-infant transmission of hepatitis B virus by immunoprophylaxis. *Chin Med J* (Engl) 2002; **115**: 1510-1512 [PMID: 12490098]
21 **Lee SD**, Lo KJ, Tsai YT, Wu JC, Wu TC, Yang ZL, Ng HT. Role of caesarean section in prevention of mother-infant transmission of hepatitis B virus. *Lancet* 1988; **2**: 833-834 [PMID: 2902274]
22 **Jhaveri R**, Murray N. An omission in the AASLD Practice Guidelines on Chronic Hepatitis B. *Hepatology* 2007; **46**: 280; author reply 280 [PMID: 17596892 DOI: 10.1002/hep.21756]
23 **Hill JB**, Sheffield JS, Kim MJ, Alexander JM, Sercely B, Wendel GD. Risk of hepatitis B transmission in breast-fed infants of chronic hepatitis B carriers. *Obstet Gynecol* 2002; **99**: 1049-1052 [PMID: 12052598]
24 **Zheng Y**, Lu Y, Ye Q, Xia Y, Zhou Y, Yao Q, Wei S. Should chronic hepatitis B mothers breastfeed? a meta analysis. *BMC Public Health* 2011; **11**: 502 [PMID: 21708016 DOI: 10.1186/1471-2458-11-502]
25 **Bzowej NH**. Hepatitis B Therapy in Pregnancy. *Curr Hepat Rep* 2010; **9**: 197-204 [PMID: 20949113 DOI: 10.1007/s11901-010-0059-x]
26 **Novartis Pharmaceuticals Corporation**. Telbivudine [Package insert]. Available from: URL: http://www.accessdata.fda.gov/drugsatfda_docs/label/2013/022011s013lbl.pdf
27 **Gilead Sciences, Inc**. Viread [Package insert]. Available from: URL: http://www.accessdata.fda.gov/drugsatfda_docs/label/2012/021356s042,022577s002lbl.pdf
28 **GlaxoSmithKline**. EPIVIR-HBV (lamivudine) Tablets EPIVIR-HBV (lamivudine) Oral Solution [Package insert]. Available from: URL: http://www.accessdata.fda.gov/drugsatfda_docs/label/2001/21004s2lbl.pdf
29 **Pyenson B**, Fitch K, Iwasaki K. Consequences of hepatitis c virus (HCV) costs of a baby boomer epidemic, 2009. Available from: URL: http://www.natap.org/2009/HCV/051809_01.htm
30 **Pergam SA**, Wang CC, Gardella CM, Sandison TG, Phipps WT, Hawes SE. Pregnancy complications associated with hepatitis C: data from a 2003-2005 Washington state birth cohort. *Am J Obstet Gynecol* 2008; **199**: 38.e1-38.e9 [PMID: 18486089 DOI: 10.1016/j.ajog.2008.03.052]
31 **Mast EE**, Hwang LY, Seto DS, Nolte FS, Nainan OV, Wurtzel H, Alter MJ. Risk factors for perinatal transmission of hepatitis C virus (HCV) and the natural history of HCV infection acquired in infancy. *J Infect Dis* 2005; **192**: 1880-1889 [PMID: 16267758 DOI: 10.1086/497701]
32 **Polywka S**, Pembrey L, Tovo PA, Newell ML. Accuracy of HCV-RNA PCR tests for diagnosis or exclusion of vertically acquired HCV infection. *J Med Virol* 2006; **78**: 305-310 [PMID: 16372293 DOI: 10.1002/jmv.20540]
33 **Gibb DM**, Goodall RL, Dunn DT, Healy M, Neave P, Cafferkey M, Butler K. Mother-to-child transmission of hepatitis C virus: evidence for preventable peripartum transmission. *Lancet* 2000; **356**: 904-907 [PMID: 11036896]
34 **Polis CB**, Shah SN, Johnson KE, Gupta A. Impact of maternal HIV coinfection on the vertical transmission of hepatitis C virus: a meta-analysis. *Clin Infect Dis* 2007; **44**: 1123-1131 [PMID: 17366462 DOI: 10.1086/512815]
35 **Mariné-Barjoan E**, Berrébi A, Giordanengo V, Favre SF, Haas H, Moreigne M, Izopet J, Tricoire J, Tran A, Pradier C, Bongain A. HCV/HIV co-infection, HCV viral load and mode of delivery: risk factors for mother-to-child transmission of hepatitis C virus? *AIDS* 2007; **21**: 1811-1815 [PMID: 17690581 DOI: 10.1097/qad.0b013e3282703810]
36 **Le Campion A**, Larouche A, Fauteux-Daniel S, Soudeyns H. Pathogenesis of hepatitis C during pregnancy and childhood. *Viruses* 2012; **4**: 3531-3550 [PMID: 23223189 DOI: 10.3390/v4123531]
37 **Thomas SL**, Newell ML, Peckham CS, Ades AE, Hall AJ. A review of hepatitis C virus (HCV) vertical transmission: risks of transmission to infants born to mothers with and without HCV viraemia or human immunodeficiency virus infection. *Int J Epidemiol* 1998; **27**: 108-117 [PMID: 9563703]
38 **Ghamar Chehreh ME**, Tabatabaei SV, Khazanehdari S, Alavian SM. Effect of cesarean section on the risk of perinatal transmission of hepatitis C virus from HCV-RNA+/HIV- mothers: a meta-analysis. *Arch Gynecol Obstet* 2011; **283**: 255-260 [PMID: 20652289 DOI: 10.1007/s00404-010-1588-9]
39 **Cottrell EB**, Chou R, Wasson N, Rahman B, Guise JM. Reducing risk for mother-to-infant transmission of hepatitis C virus: a systematic review for the U.S. Preventive Services Task Force. *Ann Intern Med* 2013; **158**: 109-113 [PMID:

23437438]
40 **Prasad MR**, Honegger JR. Hepatitis C virus in pregnancy. *Am J Perinatol* 2013; **30**: 149-159 [PMID: 23389935 DOI: 10.1055/s-0033-1334459]
41 **Dinsmoor MJ**. Hepatitis in the obstetric patient. *Infect Dis Clin North Am* 1997; **11**: 77-91 [PMID: 9067785]
42 **Khuroo MS**, Kamili S, Khuroo MS. Clinical course and duration of viremia in vertically transmitted hepatitis E virus (HEV) infection in babies born to HEV-infected mothers. *J Viral Hepat* 2009; **16**: 519-523 [PMID: 19228284 DOI: 10.1111/j.1365-2893.2009.01101.x]
43 **Sclair SN**, Schiff ER. An update on the hepatitis E virus. *Curr Gastroenterol Rep* 2013; **15**: 304 [PMID: 23314803 DOI: 10.1007/s11894-012-0304-2]
44 **Khuroo MS**, Kamili S. Aetiology, clinical course and outcome of sporadic acute viral hepatitis in pregnancy. *J Viral Hepat* 2003; **10**: 61-69 [PMID: 12558914]
45 **Boccia D**, Guthmann JP, Klovstad H, Hamid N, Tatay M, Ciglenecki I, Nizou JY, Nicand E, Guerin PJ. High mortality associated with an outbreak of hepatitis E among displaced persons in Darfur, Sudan. *Clin Infect Dis* 2006; **42**: 1679-1684 [PMID: 16705571 DOI: 10.1086/504322]
46 **Labrique AB**, Sikder SS, Krain LJ, West KP, Christian P, Rashid M, Nelson KE. Hepatitis E, a vaccine-preventable cause of maternal deaths. *Emerg Infect Dis* 2012; **18**: 1401-1404 [PMID: 22931753 DOI: 10.3201/eid1809.120241]
47 **Renou C**, Pariente A, Nicand E, Pavio N. Pathogenesis of Hepatitis E in pregnancy. *Liver Int* 2008; **28**: 1465; author reply 1466 [PMID: 19055645 DOI: 10.1111/j.1478-3231.2008.01885.x]
48 **Bhatia V**, Singhal A, Panda SK, Acharya SK. A 20-year single-center experience with acute liver failure during pregnancy: is the prognosis really worse? *Hepatology* 2008; **48**: 1577-1585 [PMID: 18925633 DOI: 10.1002/hep.22493]
49 **Borkakoti J**, Hazam RK, Mohammad A, Kumar A, Kar P. Does high viral load of hepatitis E virus influence the severity and prognosis of acute liver failure during pregnancy? *J Med Virol* 2013; **85**: 620-626 [PMID: 23280991 DOI: 10.1002/jmv.23508]
50 **Shrestha MP**, Scott RM, Joshi DM, Mammen MP, Thapa GB, Thapa N, Myint KS, Fourneau M, Kuschner RA, Shrestha SK, David MP, Seriwatana J, Vaughn DW, Safary A, Endy TP, Innis BL. Safety and efficacy of a recombinant hepatitis E vaccine. *N Engl J Med* 2007; **356**: 895-903 [PMID: 17329696 DOI: 10.1056/nejmoa061847]
51 **Krawczynski K**. Hepatitis E vaccine--ready for prime time? *N Engl J Med* 2007; **356**: 949-951 [PMID: 17329703 DOI: 10.1056/nejme068311]
52 **Zhu FC**, Zhang J, Zhang XF, Zhou C, Wang ZZ, Huang SJ, Wang H, Yang CL, Jiang HM, Cai JP, Wang YJ, Ai X, Hu YM, Tang Q, Yao X, Yan Q, Xian YL, Wu T, Li YM, Miao J, Ng MH, Shih JW, Xia NS. Efficacy and safety of a recombinant hepatitis E vaccine in healthy adults: a large-scale, randomised, double-blind placebo-controlled, phase 3 trial. *Lancet* 2010; **376**: 895-902 [PMID: 20728932 DOI: 10.1016/s0140-6736(10)61030-6]
53 **Aggarwal R**. Hepatitis E: Historical, contemporary and future perspectives. *J Gastroenterol Hepatol* 2011; **26** Suppl 1: 72-82 [PMID: 21199517 DOI: 10.1111/j.1440-1746.2010.06540.x]
54 **Kamili S**. Toward the development of a hepatitis E vaccine. *Virus Res* 2011; **161**: 93-100 [PMID: 21620908 DOI: 10.1016/j.virusres.2011.05.008]
55 **Norvell JP**, Blei AT, Jovanovic BD, Levitsky J. Herpes simplex virus hepatitis: an analysis of the published literature and institutional cases. *Liver Transpl* 2007; **13**: 1428-1434 [PMID: 17902129 DOI: 10.1002/lt.21250]
56 **Kang AH**, Graves CR. Herpes simplex hepatitis in pregnancy: a case report and review of the literature. *Obstet Gynecol Surv* 1999; **54**: 463-468 [PMID: 10394584]
57 **Peters DJ**, Greene WH, Ruggiero F, McGarrity TJ. Herpes simplex-induced fulminant hepatitis in adults: a call for empiric therapy. *Dig Dis Sci* 2000; **45**: 2399-2404 [PMID: 11258565]
58 **Riediger C**, Sauer P, Matevossian E, Müller MW, Büchler P, Friess H. Herpes simplex virus sepsis and acute liver failure. *Clin Transplant* 2009; **23** Suppl 21: 37-41 [PMID: 19930315 DOI: 10.1111/j.1399-0012.2009.01108.x]
59 **Navaneethan U**, Lancaster E, Venkatesh PG, Wang J, Neff GW. Herpes simplex virus hepatitis - it's high time we consider empiric treatment. *J Gastrointestin Liver Dis* 2011; **20**: 93-96 [PMID: 21451806]
60 **Levitsky J**, Duddempudi AT, Lakeman FD, Whitley RJ, Luby JP, Lee WM, Fontana RJ, Blei AT, Ison MG. Detection and diagnosis of herpes simplex virus infection in adults with acute liver failure. *Liver Transpl* 2008; **14**: 1498-1504 [PMID: 18825709 DOI: 10.1002/lt.21567]
61 **Beersma MF**, Verjans GM, Metselaar HJ, Osterhaus AD, Berrington WR, van Doornum GJ. Quantification of viral DNA and liver enzymes in plasma improves early diagnosis and management of herpes simplex virus hepatitis. *J Viral Hepat* 2011; **18**: e160-e166 [PMID: 20704650 DOI: 10.1111/j.1365-2893.2010.01352.x]
62 **Buchel E**, Van Steenbergen W, Nevens F, Fevery J. Improvement of autoimmune hepatitis during pregnancy followed by flare-up after delivery. *Am J Gastroenterol* 2002; **97**: 3160-3165 [PMID: 12492204 DOI: 10.1111/j.1572-0241.2002.07124.x]
63 **Candia L**, Marquez J, Espinoza LR. Autoimmune hepatitis and pregnancy: a rheumatologist's dilemma. *Semin Arthritis Rheum* 2005; **35**: 49-56 [PMID: 16084224 DOI: 10.1016/j.semarthrit.2005.03.002]
64 **Heneghan MA**, Norris SM, O'Grady JG, Harrison PM, McFarlane IG. Management and outcome of pregnancy in autoimmune hepatitis. *Gut* 2001; **48**: 97-102 [PMID: 11115829]
65 **Schramm C**, Herkel J, Beuers U, Kanzler S, Galle PR, Lohse AW. Pregnancy in autoimmune hepatitis: outcome and risk factors. *Am J Gastroenterol* 2006; **101**: 556-560 [PMID: 16464221 DOI: 10.1111/j.1572-0241.2006.00479.x]
66 **Westbrook RH**, Yeoman AD, Kriese S, Heneghan MA. Outcomes of pregnancy in women with autoimmune hepatitis. *J Autoimmun* 2012; **38**: J239-J244 [PMID: 22261501 DOI: 10.1016/j.jaut.2011.12.002]
67 **Terrabuio DR**, Abrantes-Lemos CP, Carrilho FJ, Cançado EL. Follow-up of pregnant women with autoimmune hepatitis: the disease behavior along with maternal and fetal outcomes. *J Clin Gastroenterol* 2009; **43**: 350-356 [PMID: 19077726 DOI: 10.1097/mcg.0b013e318176b8c5]
68 **Manns MP**, Czaja AJ, Gorham JD, Krawitt EL, Mieli-Vergani G, Vergani D, Vierling JM. Diagnosis and management of autoimmune hepatitis. *Hepatology* 2010; **51**: 2193-2213 [PMID: 20513004 DOI: 10.1002/hep.23584]
69 **Goh SK**, Gull SE, Alexander GJ. Pregnancy in primary biliary cirrhosis complicated by portal hypertension: report of a case and review of the literature. *BJOG* 2001; **108**: 760-762 [PMID: 11467706]
70 **Olsson R**, Lööf L, Wallerstedt S. Pregnancy in patients with primary biliary cirrhosis--a case for dissuasion? The Swedish Internal Medicine Liver Club. *Liver* 1993; **13**: 316-318 [PMID: 8295495]
71 **Lindor KD**, Gershwin ME, Poupon R, Kaplan M, Bergasa NV, Heathcote EJ. Primary biliary cirrhosis. *Hepatology* 2009; **50**: 291-308 [PMID: 19554543 DOI: 10.1002/hep.22906]
72 **Wellge BE**, Sterneck M, Teufel A, Rust C, Franke A, Schreiber S, Berg T, Günther R, Kreisel W, Zu Eulenburg C, Braun F, Beuers U, Galle PR, Lohse AW, Schramm C. Pregnancy in primary sclerosing cholangitis. *Gut* 2011; **60**: 1117-1121 [PMID: 21339205 DOI: 10.1136/gut.2010.228924]
73 **Lee NM**, Brady CW. Liver disease in pregnancy. *World J Gastroenterol* 2009; **15**: 897-906 [PMID: 19248187]
74 **Sinha S**, Taly AB, Prashanth LK, Arunodaya GR, Swamy HS. Successful pregnancies and abortions in symptomatic

and asymptomatic Wilson's disease. *J Neurol Sci* 2004; **217**: 37-40 [PMID: 14675607]
75 **Brewer GJ**, Johnson VD, Dick RD, Hedera P, Fink JK, Kluin KJ. Treatment of Wilson's disease with zinc. XVII: treatment during pregnancy. *Hepatology* 2000; **31**: 364-370 [PMID: 10655259 DOI: 10.1002/hep.510310216]
76 **Solomon L**, Abrams G, Dinner M, Berman L. Neonatal abnormalities associated with D-penicillamine treatment during pregnancy. *N Engl J Med* 1977; **296**: 54-55 [PMID: 830278]
77 **Tanaka H**, Yamanouchi M, Imai S, Hayashi Y. Low copper and brain abnormalities in fetus from triethylene tetramine dihydrochloride-treated pregnant mouse. *J Nutr Sci Vitaminol* (Tokyo) 1992; **38**: 545-554 [PMID: 1304599]
78 **Roberts EA**, Schilsky ML. Diagnosis and treatment of Wilson disease: an update. *Hepatology* 2008; **47**: 2089-2111 [PMID: 18506894 DOI: 10.1002/hep.22261]
79 **Scott LD**. Gallstone disease and pancreatitis in pregnancy. *Gastroenterol Clin North Am* 1992; **21**: 803-815 [PMID: 1478736]
80 **Everson GT**. Gastrointestinal motility in pregnancy. *Gastroenterol Clin North Am* 1992; **21**: 751-776 [PMID: 1478733]
81 **Cosenza CA**, Saffari B, Jabbour N, Stain SC, Garry D, Parekh D, Selby RR. Surgical management of biliary gallstone disease during pregnancy. *Am J Surg* 1999; **178**: 545-548 [PMID: 10670869]
82 **Al-Fozan H**, Tulandi T. Safety and risks of laparoscopy in pregnancy. *Curr Opin Obstet Gynecol* 2002; **14**: 375-379 [PMID: 12151826]
83 **Jelin EB**, Smink DS, Vernon AH, Brooks DC. Management of biliary tract disease during pregnancy: a decision analysis. *Surg Endosc* 2008; **22**: 54-60 [PMID: 17713817 DOI: 10.1007/s00464-007-9220-1]
84 **Kahaleh M**, Hartwell GD, Arseneau KO, Pajewski TN, Mullick T, Isin G, Agarwal S, Yeaton P. Safety and efficacy of ERCP in pregnancy. *Gastrointest Endosc* 2004; **60**: 287-292 [PMID: 15278066]
85 **Tang SJ**, Mayo MJ, Rodriguez-Frias E, Armstrong L, Tang L, Sreenarasimhaiah J, Lara LF, Rockey DC. Safety and utility of ERCP during pregnancy. *Gastrointest Endosc* 2009; **69**: 453-461 [PMID: 19136111 DOI: 10.1016/j.gie.2008.05.024]
86 **Russell MA**, Craigo SD. Cirrhosis and portal hypertension in pregnancy. *Semin Perinatol* 1998; **22**: 156-165 [PMID: 9638910]
87 **Tan J**, Surti B, Saab S. Pregnancy and cirrhosis. *Liver Transpl* 2008; **14**: 1081-1091 [PMID: 18668664 DOI: 10.1002/lt.21572]
88 **Sandhu BS**, Sanyal AJ. Pregnancy and liver disease. *Gastroenterol Clin North Am* 2003; **32**: 407-436, ix [PMID: 12635424]
89 **Terkivatan T**, de Wilt JH, de Man RA, Ijzermans JN. Management of hepatocellular adenoma during pregnancy. *Liver* 2000; **20**: 186-187 [PMID: 10847490]
90 **Cobey FC**, Salem RR. A review of liver masses in pregnancy and a proposed algorithm for their diagnosis and management. *Am J Surg* 2004; **187**: 181-191 [PMID: 14769302 DOI: 10.1016/j.amjsurg.2003.11.016]
91 **Noels JE**, van Aalten SM, van der Windt DJ, Kok NF, de Man RA, Terkivatan T, Ijzermans JN. Management of hepatocellular adenoma during pregnancy. *J Hepatol* 2011; **54**: 553-558 [PMID: 21094555 DOI: 10.1016/j.jhep.2010.07.022]
92 **Hill MA**, Albert T, Zieske A, Levine EA. Successful resection of multifocal hepatic adenoma during pregnancy. *South Med J* 1997; **90**: 357-361 [PMID: 9076315]
93 **Fujita S**, Kushihata F, Herrmann GE, Mergo PJ, Liu C, Nelson D, Fujikawa T, Hemming AW. Combined hepatic resection and radiofrequency ablation for multiple hepatic adenomas. *J Gastroenterol Hepatol* 2006; **21**: 1351-1354 [PMID: 16872326 DOI: 10.1111/j.1440-1746.2006.03184.x]
94 **Louie-Johnsun MW**, Hewitt PM, Perera DS, Morris DL. Fibrolamellar hepatocellular carcinoma in pregnancy. *HPB* (Oxford) 2003; **5**: 191-193 [PMID: 18332985 DOI: 10.1080/13651820310015310]
95 **Gemer O**, Segal S, Zohav E. Pregnancy in a patient with fibrolamellar hepatocellular carcinoma. *Arch Gynecol Obstet* 1994; **255**: 211-212 [PMID: 7695368]
96 **Ang CS**, Do RK, Shamseddine A, O'Reilly EM, Haydar A, Al-Olayan A, Faraj W, Boulos F, Naghy M, Makanjoula D, Farran H, Sibai H, Wehbe D, Kelsen DP, Abou-Alfa GK. A young woman with liver cancer. *Gastrointest Cancer Res* 2013; **6**: 17-21 [PMID: 23505574]
97 **Lau WY**, Leung WT, Ho S, Lam SK, Li CY, Johnson PJ, Williams R, Li AK. Hepatocellular carcinoma during pregnancy and its comparison with other pregnancy-associated malignancies. *Cancer* 1995; **75**: 2669-2676 [PMID: 7743468]
98 **Grant WJ**, McCashland T, Botha JF, Shaw BW, Sudan DL, Mejia A, Iyer K, Langnas AN. Acute Budd-Chiari syndrome during pregnancy: surgical treatment and orthotopic liver transplantation with successful completion of the pregnancy. *Liver Transpl* 2003; **9**: 976-979 [PMID: 12942460 DOI: 10.1053/jlts.2003.50134]
99 **Fickert P**, Ramschak H, Kenner L, Hoefler G, Hinterleitner TA, Petritsch W, Klimpfinger M, Krejs GJ, Stauber RE. Acute Budd-Chiari syndrome with fulminant hepatic failure in a pregnant woman with factor V Leiden mutation. *Gastroenterology* 1996; **111**: 1670-1673 [PMID: 8942748]
100 **DeLeve LD**, Valla DC, Garcia-Tsao G. Vascular disorders of the liver. *Hepatology* 2009; **49**: 1729-1764 [PMID: 19399912 DOI: 10.1002/hep.22772]

P- Reviewers: Devarbhavi H, Invernizzi P, Morales-Gonzalez JA, Rigato I **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.v19.i43.7639

World J Gastroenterol 2013 November 21; 19(43): 7639-7646
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

MINIREVIEWS

Liver diseases in pregnancy: Diseases unique to pregnancy

Khulood T Ahmed, Ashraf A Almashhrawi, Rubayat N Rahman, Ghassan M Hammoud, Jamal A Ibdah

Khulood T Ahmed, Ashraf A Almashhrawi, Rubayat N Rahman, Ghassan M Hammoud, Jamal A Ibdah, Division of Gastroenterology and Hepatology, University of Missouri-Columbia, Columbia, MO 65212, United States

Author contributions: Ahmed KT wrote and revised the manuscript; Almashhrawi AA, Rahman RN, and Hammoud GM were involved in reviewing the literature and collecting data; and Ibdah JA conceived the topic, contributed to the writing, analyzed and edited the manuscript, and provided overall intellectual input into the design and execution of the manuscript.

Correspondence to: Jamal A Ibdah, MD, PhD, Professor, Director, Division of Gastroenterology and Hepatology, University of Missouri-Columbia, 319 jesse hall, Columbia, MO 65212, United States. ibdahj@health.missouri.edu

Telephone: +1-573-8827349 Fax: +1-573-8844595
Received: June 10, 2013 Revised: August 5, 2013
Accepted: September 4, 2013
Published online: November 21, 2013

Abstract

Pregnancy is a special clinical state with several normal physiological changes that influence body organs including the liver. Liver disease can cause significant morbidity and mortality in both pregnant women and their infants. This review summarizes liver diseases that are unique to pregnancy. We discuss clinical conditions that are seen only in pregnant women and involve the liver; from Hyperemesis Gravidarum that happens in 1 out of 200 pregnancies and Intrahepatic Cholestasis of Pregnancy (0.5%-1.5% prevalence), to the more frequent condition of preeclampsia (10% prevalence) and its severe form; hemolysis, elevated liver enzymes, and a low platelet count syndrome (12% of pregnancies with preeclampsia), to the rare entity of Acute Fatty Liver of Pregnancy (incidence of 1 per 7270 to 13000 deliveries). Although pathogeneses behind the development of these aliments are not fully understood, theories have been proposed. Some propose the special physiological changes that accompany pregnancy as a precipitant. Others suggest a constellation of factors including both the mother and her fetus that come together to trigger those unique conditions. Reaching a timely and accurate diagnosis of such conditions can be challenging. The timing of the condition in relation toward which trimester it starts at is a key. Accurate diagnosis can be made using specific clinical findings and blood tests. Some entities have well-defined criteria that help not only in making the diagnosis, but also in classifying the disease according to its severity. Management of these conditions range from simple medical remedies to measures such as immediate termination of the pregnancy. In specific conditions, it is prudent to have expert obstetric and medical specialists teaming up to help improve the outcomes.

Key words: Liver; Pregnancy; Hyperemesis gravidarum; Intrahepatic cholestasis; Hemolysis, elevated liver enzymes, and a low platelet count; Preeclampsia; Eclampsia; Acute fatty liver

Core tip: Pregnancy is a special clinical state with several normal physiological changes that influence body organs including the liver. Liver disease can cause significant morbidity and mortality in both pregnant women and their infants. Challenges involve making the diagnosis and the methods of treatment and their safety for both the mother and the baby. This review summarizes liver diseases that are unique to pregnancy.

Ahmed KT, Almashhrawi AA, Rahman RN, Hammoud GM, Ibdah JA. Liver diseases in pregnancy: Diseases unique to pregnancy. *World J Gastroenterol* 2013; 19(43): 7639-7646 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7639.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7639

HYPEREMESIS GRAVIDARUM

Although nausea and vomiting of pregnancy affect up to 90% of pregnancies, hyperemesis gravidarum (HG) oc-

curs in approximately 1 out of every 200 pregnancies[1]. Women with HG present with severe and persistent vomiting in the first trimester that can cause dehydration, metabolic disturbances, and nutritional deficiencies. HG may result in weight loss and ketonuria. Risk factors for HG include multiple gestations, molar pregnancies, fetal anomalies such as hydrops fetalis and trisomy 21[2,3]. Not all women with HG develop liver disease. Half of the patients who require hospitalization for HG suffer from liver disease[4]. HG was the cause in up to 94% of pregnant women with elevated liver transaminases in their first trimester in one series[5]. Veenendaal *et al*[6] conducted a meta-analysis that showed women with HG are more likely to have low birthweight < 2500 kg (OR = 1.42; 95%CI: 1.27-1.58), small for gestational age (OR = 1.28; 95%CI: 1.02-1.60), and premature delivery (OR = 1.32; 95%CI: 1.04-1.68) than those with no HG. On the other hand, no correlations with Apgar scores, congenital anomalies or perinatal death were identified. Some of those poor outcomes were more likely in pregnant women with low gestational weight gain (< 7 kg)[7].

Pathogenesis

Despite several hypotheses, the pathogenesis of liver disease in HG is not well understood and likely multifactorial. Starvation injury was proposed as an etiology since 1968[8,9]. Over expression of cytokine-producing cells was implicated as a potential cause for pregnancy-related liver diseases such as preeclampsia and HG. Other hypotheses predicted damage to the liver resulting from impaired maternal or fetal mitochondrial fatty acid oxidation, implicating deficiency in long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) as a reason for accumulation of fatty acids in the placenta and eventually causing liver damage[10]. Other report linked fetal deficiency of hepatic carnitine palmitoyltransferase I , the enzyme responsible for transporting long chain fatty acids from the cytoplasm of cells across the outer mitochondrial membrane, to HG[11-16].

Clinical presentation

The clinical presentation of HG with liver disease can range from mild aminotransferase elevation to rarely severe elevation. No fulminant hepatic failure has been reported with HG[17,18]. Patients usually are acutely ill with signs of dehydration. Rarely, it can present with jaundice and electrolyte disturbances such as hypokalemia and hyponatremia as well as metabolic alkalosis and erythrocytosis. It seems that the severity of nausea and vomiting correlates well with the degree of liver enzymes elevation[4]. No specific abdominal ultrasound findings are associated with HG. Liver biopsy may show necrosis, steatosis or bile plugs[19,20], and usually is not indicated.

Management

Patients with HG usually require hospitalization for intravenous fluid replacement, anti-emetics, bowel rest, and possible parenteral nutrition.

Prognosis

Hyperemesis gravidarum is usually a reversible condition with no permanent damage to the liver and almost never fatal.

INTRAHEPATIC CHOLESTASIS OF PREGNANCY

Intrahepatic cholestasis of pregnancy (ICP) is a reversible condition of cholestasis that happens usually in the third trimester. Findings such as pruritus, high serum bile acids levels, and abnormal liver function tests usually resolve after delivery. ICP is more prevalent in Scandinavian and South American countries[21,22]. Prevalence in Europe, United States, Canada and Australia is 0.1% to 1.5%[23]. In a recent review, although no causality effect can be claimed, ICP was associated with an increase in the risk of developing hepatobiliary diseases later in life, such as hepatitis C, cirrhosis, and gallstones. Having underlying chronic liver disease (hepatitis C or chronic hepatitis) increased the odds of developing ICP[24].

Pathogenesis

Genetic predisposition and hormonal factors have been implicated in the pathogenesis of ICP. The familial tendency and the observation of clustering of ICP in families led to the belief that genetics play a role in its development. Although some studies revealed results connecting MDR3 (*ABCB4*) gene with ICP, several other studies failed to demonstrate such relation[25-29]. Other genes such as *ABCB11* and *ATP8B1* were examined but showed weaker linkage to ICP[30-32]. Explaining ICP on a molecular basis in relation to sex hormones has gained interest[33]. The facts that ICP happens late in pregnancy and has a higher incidence in multiple gestation pregnancies, and that it resolves after delivery when sex hormones levels fall, make a logical connection between sex hormones and ICP. The estrogen metabolite estradiol-17β-glucuronide and differences in progesterone metabolites between pregnant women with and without ICP were also implicated[34-38].

Clinical presentation

ICP usually commences in the third trimester although earlier start in the second trimester has been reported[39]. The most common symptom is pruritus. Severity of pruritus increases at night and can involve the palms and soles. Other symptoms include steatorrhea, malabsorption of fat-soluble vitamins, and weight loss. ICP seems also to increase the incidence of gallstones and cholecystitis[40]. ICP tends to return in subsequent pregnancies with variable severity[41]. Elevated fasting serum bile acids level (> 10 μmol/L) confirms the diagnosis. Aminotransferases can be elevated as well up to 2-10 folds[42]. Alkaline phosphatase levels might not be helpful due to higher physiological levels in late pregnancy. Clinical jaundice is detected in 10%-15% of the cases only and bilirubin levels rarely exceed 100 μmol/L[23,43]. As in all

cholestatic patients, women with ICP tend to have higher low-density lipoprotein cholesterol and triglycerides[44]. Liver biopsy can reveal bland cholestasis (intrahepatic cholestasis without parenchymal inflammation). Liver biopsy is usually not indicated.

Management

Bile acids sequestrants such as cholestyramine, antihistamines and opioid antagonists have been used to alleviate the pruritus. Cholestyramine is an exchange resin that binds bile acids and other anions in the intestine and increases their fecal excretion. Cholestyramine does not improve biochemical parameters or fetal outcomes in ICP[45]. *S*-adenosyl-methionine has shown limited efficacy in ICP[46,47]. Ursodeoxycholic acid (UDCA) is the first line therapy for ICP. UDCA has shown significant decrease in serum bile acids, serum aspartate aminotransferase and alanine aminotransferase, serum bilirubin, and was effective for pruritus[48-50]. Weekly non-stress testing did not prove to make a difference in ICP-related fetal deaths[51]. Some studies suggested 40 μmol/L as a cutoff level of bile acids, after that fetal complications may happen[52,53]. Others did not observe such correlation until bile acids are > 100 μmol/L[54]. No evidence is strong enough to recommend early delivery (at 37 wk of gestation) for mothers with high bile acids levels, although this strategy is still used in some practices[55].

Prognosis

Although ICP is a benign condition for the mother, poor fetal outcomes can occur. In some studies ICP resulted in premature births up to 60%. Other complications such as fetal distress and intrauterine fetal death were reported at 61% and 1.6% respectively[23,39,56]. The onset of pruritus and higher maternal fasting serum bile acids were associated with higher risk for premature delivery[57].

ACUTE FATTY LIVER OF PREGNANCY

Acute fatty liver of pregnancy (AFLP) is a rare but a serious condition that is unique to pregnancy and happens in the third trimester. AFLP can lead to significant maternal and fetal morbidity and mortality[20,58]. Although rare, incidence of 1 per 7270 to 13000 deliveries, outcomes can be grave with acute liver failure and death[20,59-61].

Pathogenesis

Until recently the pathogenesis of AFLP was unknown and still has not been fully elucidated. However, molecular advances over the past decade suggest that AFLP may result from mitochondrial dysfunction. Defects in fetal mitochondrial fatty acid β-oxidation have been linked to development of maternal AFLP, particularly fetal defects in LCHAD, which is part of the mitochondrial trifunctional protein (MTP) complex[14,62-66]. In a retrospective study, Ibdah *et al*[14] examined the association between MTP defects in children and liver disease in their mothers during pregnancy in 24 families with documented pediatric defects in MTP. Fifteen of 24 women (62%) were diagnosed to have had maternal liver disease consistent with AFLP, although in two cases a clear distinction between AFLP and hemolysis, elevated liver enzymes, and a low platelet count (HELLP) syndrome was not possible. Nine of the 24 women had normal pregnancies. All 15 pregnancies with maternal liver disease were associated with fetal LCHAD deficiency. Molecular analysis revealed a common LCHAD mutation, G1528C in the offspring of women who developed AFLP. The results from this study show that when carrying a fetus that is LCHAD deficient, the mother has a high risk of developing AFLP. In a subsequent study, Ibdah et al evaluated fetal genotypes and pregnancy outcomes in 83 pregnancies in 35 families with documented pediatric MTP defects[66]. This study provided further evidence that carrying a fetus with LCHAD deficiency is associated with a high risk for developing AFLP. With the growing evidence suggesting that carrying an LCHAD-deficient fetus is associated with AFLP, it was recommended that neonates born to pregnancies complicated by AFLP be tested for the common G1528C mutation and that this testing when done early after birth can be lifesaving as it may identify LCHAD-deficient children before they manifest the disease allowing early dietary intervention by institution of a diet low in fat, high in carbohydrate, and by substitution of the long chain fatty acids with medium chain fatty acids (for complete review on the association between AFLP and pediatric LCHAD deficiency[61]).

The precise mechanism by which an LCHAD deficient fetus causes AFLP in a heterozygote mother is still unclear. However, several factors appear to contribute to this fetal-maternal interaction. First, the heterozygosity of the mother for an MTP defect reduces her capacity to oxidize long chain fatty acids. Second, third trimester is accompanied by changes in metabolism, an increased lipolysis, and a reduction in mitochondrial fatty acid oxidation, all increase the susceptibility of the mother who carries a fetus with LCHAD deficiency. Thus it has been speculated that potentially hepatotoxic long-chain 3-hydroxyacyl fatty acid metabolites, produced by the affected fetus or placenta, accumulate in the maternal circulation[61].

Clinical presentation

Although there were few reports of AFLP starting in the second trimester, it usually presents in the third trimester between the 30th and 38th week of gestation[67-70]. It is more frequent in primiparous women and can return in subsequent pregnancies[12,62,71]. Nonspecific symptoms such as nausea, vomiting, headache, and fatigue can be the initial presentation. Right upper quadrant pain or epigastric pain can occur. Jaundice common and early jaundice may indicate severe disease[72]. Other features such as hypoglycemia, renal failure, coagulopathy, ascites, and encephalopathy were reported frequently. AFLP can result in maternal and fetal demise[73]. Although hypertension can be present, severe hypertension is likely

Table 1 Proposed (Swansea) diagnostic criteria for acute fatty liver of pregnancy

Vomiting	Abdominal pain
Polydipsia/polyuria	Encephalopathy
Elevated bilirubin	Hypoglycaemia
Elevated uric acid	Leucocytosis
Ascites or bright liver on US	Elevated transaminases
Elevated ammonia	Renal impairment
Coagulopathy	Microvesicular steatosis on liver biopsy

To meet the criteria the patient should have 6 or more of these clinical findings. Source: Ref. [80], with permission; US: Ultrasound scan.

secondary to the reduction in peripheral vascular resistance associated with liver failure. AFLP is a medical and obstetric emergency and diagnosis relying on clinical and laboratory findings should be prompt. Liver biopsy can be helpful in early and mild cases of AFLP especially if diagnosis is not clear[74]. Liver biopsy is not necessarily needed and should be avoided in more severe cases were the risk of bleeding is high and prompt therapeutic intervention is needed. Although elevated aminotransferases is expected, the severity of liver dysfunction is not always reflected by the degree of elevation. Alkaline phosphatase is usually elevated. Other findings such as leukocytosis, thrombocytopenia, disseminated intravascular coagulopathy (DIC), abnormal prothrombin time, partial thromboplastin time, and normal fibrinogen can occur[74-76]. Ketonuria and proteinuria can be present. Elevated blood urea nitrogen and creatinine indicate renal insufficiency. Low serum albumin and hypoglycemia can occur. Uric acid and ammonia levels can be increased. Hyperuricemia can be an early indicator and develop before hyperbilirubinemia[77,78]. In comparison with diffuse or microvesicular steatosis, Swansea criteria had a sensitivity of 100% (95%CI: 77-100) and specificity of 57% (95%CI: 20-88), with positive and negative predictive values of 85% and 100% in one report (Table 1)[79-81]. Ch'ng *et al*[80] proposed a set of clinical findings, known as Swansea criteria, to help reach the diagnosis of AFLP. Those diagnostic criteria have not been validated in different populations. Liver biopsy usually displays microvesicular steatosis[82]. Electron microscopy can show mitochondrial disruption. Imaging studies can be useful to exclude other pathologies; but have limited utility in the diagnosis of AFLP.

Management

Stabilization of the mother and early recognition and delivery are the keys for successful management. Close monitoring and management of associated complications is necessary to improve outcomes. Plasmapheresis was used in few series in severe cases with reported success[83,84].

Prognosis

AFLP is severe disease with high maternal (18%) and fetal (23%) mortality. Prenatal diagnosis can provide benefit for both the mother and her fetus in subsequent pregnancies.

Table 2 Hemolysis, elevated liver function tests, and low platelet counts syndrome diagnostic criteria

HELLP class	Tennessee classification	Mississippi classification
1	Platelets $\leq 100 \times 10^9$/L AST $\geq$ 70 IU/L LDH $\geq$ 600 IU/L	Platelets $\leq 50 \times 10^9$/L AST or ALT $\geq$ 70 IU/L LDH $\geq$ 600 IU/L
2		Platelets $\leq 100 \times 10^9$/L, $\geq 50 \times 10^9$/L AST or ALT $\geq$ 70 IU/L LDH $\geq$ 600 IU/L
3		Platelets $\leq 150 \times 10^9$/L, $\geq 100 \times 10^9$/L AST or ALT $\geq$ 40 IU/L LDH $\geq$ 600 IU/L

AST: Aspartate aminotransferase; Source: Haram *et al*. BMC Pregnancy and Childbirth 2009 9:8 doi:10.1186/1471-2393-9-8; ALT: Alanine aminotransferase; HELLP: Hemolysis, elevated liver function tests, and low platelet counts; LDH: Lactate dehydrogenase.

PREECLAMPSIA/ECLAMPSIA AND HELLP SYNDROME

Preeclampsia is a syndrome that is unique to pregnancy. Manifestations include hypertension and proteinuria, and can result in fetal growth retardation. By far, preeclampsia is the most common serious medical disorder in pregnancy with prevalence up to 10%. It is associated with up to 20% of maternal mortality in developed countries[85,86]. Organ involvement such as liver, brain and kidneys signifies severe disease. Elevated aminotransferases occurs up to 10% of severe preeclampsia cases[86,87]. Although preeclampsia can start as early as the second trimester, liver involvement is mainly seen in the third trimester. Severe preeclampsia can be life threatening to the mother and can result in fetal morbidity and mortality. Eclampsia usually refers to preeclampsia with seizures. HELLP syndrome is a variant of severe preeclampsia that happens in up to 12% of patients with preeclampsia, and entails constellation of findings including hemolysis, elevated liver aminotransferases of and low platelet counts. Table 2 shows the diagnostic criteria of HELLP syndrome.

Pathogenesis

In reviewing liver biopsies and autopsies of cases with preeclampsia, eclampsia and unclassified toxemia, from the Armed Forces Institute of Pathology between 1920 and 1984, Rolfes *et al*[88] reported that despite that large cerebral and midbrain hemorrhages, extensive thrombosis and infarction as well as cerebral edema with herniation were the major causes of deaths, liver disease contributed to 17 deaths out of the 102 cases reviewed. Extensive periportal lesions producing widespread parenchymal hemorrhage and necrosis were described. Large areas of infarction, wide bands of fibrin replacing liver cells, extravasation of red blood cells, and capillary

Table 3 Preeclampsia associated liver diseases

	Severe preeclampsia and eclampsia	HELLP syndrome	Acute fatty liver of pregnancy
Time	After gestational week 22	Late second trimester to early postpartum	Third trimester
Prevalence	Increases in multiple gestation (5%-7%)	0.10%	Increases in male fetus, multiple gestations, primiparous women (0.01%)
Findings	High blood pressure; proteinuria; edema; seizure; renal failure; pulmonary edema	Abdominal pain, nausea/vomiting, overlap with findings in preeclampsia	Abdominal pain, nausea/ vomiting, jaundice, hypoglycemia and hepatic failure
Tests	Platelets > 70000; urine protein > 5 g/24 h; abnormal liver enzymes (10%)	Low platelets; hemolysis; elevated liver enzymes; prothrombin time may remain normal; normal fibrinogen	Platelets < 100000; AST and ALT 300-1000 U/L; low antithrombin Ⅲ; high prothrombin time; low fibrinogen; high bilirubin; DIC
Management	Blood pressure control; beta-blockers, methyldopa, magnesium sulfate, early delivery	Prompt delivery 5% maternal death 1% hepatic rupture	Prompt delivery; liver transplant ⩽ 10% maternal death
Outcome	1% maternal death	1%-30% fetal death	Up to 45% fetal death

HELLP: Hemolysis, elevated liver function tests, and low platelet counts; DIC: disseminated intravascular coagulation; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

Table 4 Complications of preeclampsia/hemolysis, elevated liver function tests, and low platelet counts syndrome

Maternal complications	Neonatal complications	Labor complications
Eclampsia	Fetal death	Preterm labor
HELLP syndrome	Prematurity	
Hepatic subcapsular hematoma, infarction or rupture	IUGR	
Acute renal failure	Respiratory distress syndrome	
Stroke, cerebral hemorrhage, edema and herniation	Intraventricular hemorrhage	
Pulmonary edema and acute respiratory distress syndrome	Sepsis	
Laryngeal edema		
Retinal detachment		

HELLP: Hemolysis, elevated liver function tests, and low platelet counts; IUGR: Intrauterine growth retardation.

thrombi were also seen. Histological changes of the liver in HELLP syndrome include periportal or focal parenchymal necrosis with hyaline deposits of fibrin-like material in the sinusoids[89]. Other molecular mechanisms such as vascular remodeling and placentation, immunological factors, and fatty acid oxidation defects were proposed as potential factors in the development of this spectrum of diseases[12,71,90-92].

Clinical presentation

Preeclampsia, HELLP syndrome, and acute fatty liver of pregnancy share similar presentations and differentiating between the three entities can be difficult. All present late in pregnancy and can have similar clinical features. Clinical presentation followed by typical laboratory findings can help in reaching the diagnosis. Although it may be reasonable to do an ultrasound of the liver for pregnant women with abnormal liver enzymes, imaging studies such as computed tomography and magnetic resonance imaging are rarely useful in making the diagnosis. Such studies can have a role in diagnosing complications such as liver infarcts, hematomas, and liver rupture[93]. Table 3 presents a comparison between the three preeclampsia-associated liver diseases in pregnancy.

Management

Successful management strategies rely on early diagnosis and prompt intervention. Women with severe preeclampsia or HELLP syndrome should be hospitalized and closely monitored in labor and delivery units, and placed on bed rest with good blood pressure control (systolic blood pressure < 155 and diastolic blood pressure < 100)[94]. The use of intravenous magnesium sulfate to prevent seizures is recommended. Close monitoring of mental status and appropriate use of imaging studies as indicated can help in identifying complications early. Prompt delivery can be the only effective therapy. Timing of delivery should be based on gestational age (reflecting the degree of fetal maturity) and the severity of the disease (maternal morbidity and mortality). Prompt delivery is indicated if the syndrome develops after 34 wk of gestation or earlier if complications occur, such as multi-organ dysfunction, liver infarction or hemorrhage, DIC, renal failure, suspected abruption of placenta, or fetal compromise[95-97]. Fetal lung maturity is not achieved before 34 wk of gestation. Therefore making a determination about terminating the pregnancy before 34 wk of gestation can be difficult[96,98-102]. Although a favorable effect on the platelet count and the aminotransferases levels has been observed, it's not clear if corticosteroids alter the course of the disease, and therefore their use remains controversial[101,103,104]. Betamethasone 12 mg intramuscularly every 24 h twice or four doses of intramuscular dexamethasone 6 mg every 12 h is recommended for enhancing fetal maturity[101]. Fetal and maternal complications are listed in Table 4.

Prognosis

Although not very common, preeclampsia and HELLP syndrome remain a significant cause of morbidity and mortality for both pregnant women and their fetuses. With a maternal mortality of 1% in severe preeclamp-

sia, up to 5% in HELLP syndrome, and up to 30% fetal death rate, early diagnosis and prompt delivery remain the only effective treatment strategy.

REFERENCES

1 **Weigel MM**, Weigel RM. Nausea and vomiting of early pregnancy and pregnancy outcome. An epidemiological study. *Br J Obstet Gynaecol* 1989; **96**: 1304-1311 [PMID: 2611169]
2 **Philip B**. Hyperemesis gravidarum: literature review. *WMJ* 2003; **102**: 46-51 [PMID: 12822290]
3 **Koch KL**, Frissora CL. Nausea and vomiting during pregnancy. *Gastroenterol Clin North Am* 2003; **32**: 201-234, vi [PMID: 12635417]
4 **Abell TL**, Riely CA. Hyperemesis gravidarum. *Gastroenterol Clin North Am* 1992; **21**: 835-849 [PMID: 1478739]
5 **Wong HY**, Tan JY, Lim CC. Abnormal liver function tests in the symptomatic pregnant patient: the local experience in Singapore. *Ann Acad Med Singapore* 2004; **33**: 204-208 [PMID: 15098635]
6 **Veenendaal MV**, van Abeelen AF, Painter RC, van der Post JA, Roseboom TJ. Consequences of hyperemesis gravidarum for offspring: a systematic review and meta-analysis. *BJOG* 2011; **118**: 1302-1313 [PMID: 21749625 DOI: 10.1111/j.1471-0528.2011.03023.x]
7 **Dodds L**, Fell DB, Joseph KS, Allen VM, Butler B. Outcomes of pregnancies complicated by hyperemesis gravidarum. *Obstet Gynecol* 2006; **107**: 285-292 [PMID: 16449113 DOI: 10.1097/01.aog.0000195060.22832.cd]
8 **Morali GA**, Braverman DZ. Abnormal liver enzymes and ketonuria in hyperemesis gravidarum. A retrospective review of 80 patients. *J Clin Gastroenterol* 1990; **12**: 303-305 [PMID: 2362099]
9 **Adams RH**, Gordon J, Combes B. Hyperemesis gravidarum. I. Evidence of hepatic dysfunction. *Obstet Gynecol* 1968; **31**: 659-664 [PMID: 5646397]
10 **Outlaw WM**, Ibdah JA. Impaired fatty acid oxidation as a cause of liver disease associated with hyperemesis gravidarum. *Med Hypotheses* 2005; **65**: 1150-1153 [PMID: 16040200 DOI: 10.1016/j.mehy.2005.05.035]
11 **Ibdah JA**, Yang Z, Bennett MJ. Liver disease in pregnancy and fetal fatty acid oxidation defects. *Mol Genet Metab* 2000; **71**: 182-189 [PMID: 11001809 DOI: 10.1006/mgme.2000.3065]
12 **Schoeman MN**, Batey RG, Wilcken B. Recurrent acute fatty liver of pregnancy associated with a fatty-acid oxidation defect in the offspring. *Gastroenterology* 1991; **100**: 544-548 [PMID: 1985050]
13 **Tyni T**, Ekholm E, Pihko H. Pregnancy complications are frequent in long-chain 3-hydroxyacyl-coenzyme A dehydrogenase deficiency. *Am J Obstet Gynecol* 1998; **178**: 603-608 [PMID: 9539533]
14 **Ibdah JA**, Bennett MJ, Rinaldo P, Zhao Y, Gibson B, Sims HF, Strauss AW. A fetal fatty-acid oxidation disorder as a cause of liver disease in pregnant women. *N Engl J Med* 1999; **340**: 1723-1731 [PMID: 10352164 DOI: 10.1056/nejm199906033402204]
15 **Innes AM**, Seargeant LE, Balachandra K, Roe CR, Wanders RJ, Ruiter JP, Casiro O, Grewar DA, Greenberg CR. Hepatic carnitine palmitoyltransferase I deficiency presenting as maternal illness in pregnancy. *Pediatr Res* 2000; **47**: 43-45 [PMID: 10625081]
16 **Shekhawat P**, Bennett MJ, Sadovsky Y, Nelson DM, Rakheja D, Strauss AW. Human placenta metabolizes fatty acids: implications for fetal fatty acid oxidation disorders and maternal liver diseases. *Am J Physiol Endocrinol Metab* 2003; **284**: E1098-E1105 [PMID: 12582009 DOI: 10.1152/ajpendo.00481.2002]
17 **Conchillo JM**, Koek GH. Hyperemesis gravidarum and severe liver enzyme elevation. *J Hepatol* 2002; **37**: 162-163 [PMID: 12076879]
18 **Orazi G**, Dufour PH, Puech F. Jaundice induced by hyperemesis gravidarum. *Int J Gynaecol Obstet* 1998; **61**: 181-183 [PMID: 9639224]
19 **Larrey D**, Rueff B, Feldmann G, Degott C, Danan G, Benhamou JP. Recurrent jaundice caused by recurrent hyperemesis gravidarum. *Gut* 1984; **25**: 1414-1415 [PMID: 6510771]
20 **Knox TA**, Olans LB. Liver disease in pregnancy. *N Engl J Med* 1996; **335**: 569-576 [PMID: 8678935 DOI: 10.1056/nejm199608223350807]
21 **Reyes H**, Gonzalez MC, Ribalta J, Aburto H, Matus C, Schramm G, Katz R, Medina E. Prevalence of intrahepatic cholestasis of pregnancy in Chile. *Ann Intern Med* 1978; **88**: 487-493 [PMID: 637428]
22 **Reyes H**, Taboada G, Ribalta J. Prevalence of intrahepatic cholestasis of pregnancy in La Paz, Bolivia. *J Chronic Dis* 1979; **32**: 499-504 [PMID: 457835]
23 **Geenes V**, Williamson C. Intrahepatic cholestasis of pregnancy. *World J Gastroenterol* 2009; **15**: 2049-2066 [PMID: 19418576]
24 **Marschall HU**, Wikström Shemer E, Ludvigsson JF, Stephansson O. Intrahepatic cholestasis of pregnancy and associated hepatobiliary disease: A population-based cohort study. *Hepatology* 2013; **58**: 1385-1391 [PMID: 23564560 DOI: 10.1002/hep.26444]
25 **Savander M**, Ropponen A, Avela K, Weerasekera N, Cormand B, Hirvioja ML, Riikonen S, Ylikorkala O, Lehesjoki AE, Williamson C, Aittomäki K. Genetic evidence of heterogeneity in intrahepatic cholestasis of pregnancy. *Gut* 2003; **52**: 1025-1029 [PMID: 12801961]
26 **Müllenbach R**, Linton KJ, Wiltshire S, Weerasekera N, Chambers J, Elias E, Higgins CF, Johnston DG, McCarthy MI, Williamson C. ABCB4 gene sequence variation in women with intrahepatic cholestasis of pregnancy. *J Med Genet* 2003; **40**: e70 [PMID: 12746424]
27 **Gendrot C**, Bacq Y, Brechot MC, Lansac J, Andres C. A second heterozygous MDR3 nonsense mutation associated with intrahepatic cholestasis of pregnancy. *J Med Genet* 2003; **40**: e32 [PMID: 12624161]
28 **Dixon PH**, Weerasekera N, Linton KJ, Donaldson O, Chambers J, Egginton E, Weaver J, Nelson-Piercy C, de Swiet M, Warnes G, Elias E, Higgins CF, Johnston DG, McCarthy MI, Williamson C. Heterozygous MDR3 missense mutation associated with intrahepatic cholestasis of pregnancy: evidence for a defect in protein trafficking. *Hum Mol Genet* 2000; **9**: 1209-1217 [PMID: 10767346]
29 **Bacq Y**, Gendrot C, Perrotin F, Lefrou L, Chrétien S, Vie-Buret V, Brechot MC, Andres CR. ABCB4 gene mutations and single-nucleotide polymorphisms in women with intrahepatic cholestasis of pregnancy. *J Med Genet* 2009; **46**: 711-715 [PMID: 19584064 DOI: 10.1136/jmg.2009.067397]
30 **Dixon PH**, van Mil SW, Chambers J, Strautnieks S, Thompson RJ, Lammert F, Kubitz R, Keitel V, Glantz A, Mattsson LA, Marschall HU, Molokhia M, Moore GE, Linton KJ, Williamson C. Contribution of variant alleles of ABCB11 to susceptibility to intrahepatic cholestasis of pregnancy. *Gut* 2009; **58**: 537-544 [PMID: 18987030 DOI: 10.1136/gut.2008.159541]
31 **Müllenbach R**, Bennett A, Tetlow N, Patel N, Hamilton G, Cheng F, Chambers J, Howard R, Taylor-Robinson SD, Williamson C. ATP8B1 mutations in British cases with intrahepatic cholestasis of pregnancy. *Gut* 2005; **54**: 829-834 [PMID: 15888793 DOI: 10.1136/gut.2004.058115]
32 **Painter JN**, Savander M, Ropponen A, Nupponen N, Riikonen S, Ylikorkala O, Lehesjoki AE, Aittomäki K. Sequence variation in the ATP8B1 gene and intrahepatic cholestasis of pregnancy. *Eur J Hum Genet* 2005; **13**: 435-439 [PMID: 15657619 DOI: 10.1038/sj.ejhg.5201355]
33 **Kreek MJ**. Female sex steroids and cholestasis. *Semin Liver Dis* 1987; **7**: 8-23 [PMID: 3296217 DOI: 10.1055/s-2008-1040559]

34 **Reyes H**, Sjövall J. Bile acids and progesterone metabolites in intrahepatic cholestasis of pregnancy. *Ann Med* 2000; **32**: 94-106 [PMID: 10766400]

35 **Trauner M**, Arrese M, Soroka CJ, Ananthanarayanan M, Koeppel TA, Schlosser SF, Suchy FJ, Keppler D, Boyer JL. The rat canalicular conjugate export pump (Mrp2) is down-regulated in intrahepatic and obstructive cholestasis. *Gastroenterology* 1997; **113**: 255-264 [PMID: 9207286]

36 **Stieger B**, Fattinger K, Madon J, Kullak-Ublick GA, Meier PJ. Drug- and estrogen-induced cholestasis through inhibition of the hepatocellular bile salt export pump (Bsep) of rat liver. *Gastroenterology* 2000; **118**: 422-430 [PMID: 10648470]

37 **Giusti G**, Piccinino F, Ricciardi I, Delrio G, Sagnelli E, Manzillo G. Abnormal steroid sulfate in plasma of women with intrahepatic cholestasis of pregnancy. *Acta Hepatogastroenterol* (Stuttg) 1979; **26**: 203-206 [PMID: 484169]

38 **Arrese M**, Macias RI, Briz O, Perez MJ, Marin JJ. Molecular pathogenesis of intrahepatic cholestasis of pregnancy. *Expert Rev Mol Med* 2008; **10**: e9 [PMID: 18371245 DOI: 10.1017/s1462399408000628]

39 **Lammert F**, Marschall HU, Glantz A, Matern S. Intrahepatic cholestasis of pregnancy: molecular pathogenesis, diagnosis and management. *J Hepatol* 2000; **33**: 1012-1021 [PMID: 11131439]

40 **Ropponen A**, Sund R, Riikonen S, Ylikorkala O, Aittomäki K. Intrahepatic cholestasis of pregnancy as an indicator of liver and biliary diseases: a population-based study. *Hepatology* 2006; **43**: 723-728 [PMID: 16557542 DOI: 10.1002/hep.21111]

41 **Bacq Y**, Sapey T, Bréchot MC, Pierre F, Fignon A, Dubois F. Intrahepatic cholestasis of pregnancy: a French prospective study. *Hepatology* 1997; **26**: 358-364 [PMID: 9252146 DOI: 10.1002/hep.510260216]

42 **Lunzer M**, Barnes P, Byth K, O'Halloran M. Serum bile acid concentrations during pregnancy and their relationship to obstetric cholestasis. *Gastroenterology* 1986; **91**: 825-829 [PMID: 3743960]

43 **Bacq Y**. Liver diseases unique to pregnancy: a 2010 update. *Clin Res Hepatol Gastroenterol* 2011; **35**: 182-193 [PMID: 21310683 DOI: 10.1016/j.clinre.2010.11.011]

44 **IBER FL**. JAUNDICE IN PREGNANCY--A REVIEW. *Am J Obstet Gynecol* 1965; **91**: 721-753 [PMID: 14268788]

45 **Bergasa NV**. The pruritus of cholestasis. *Semin Dermatol* 1995; **14**: 302-312 [PMID: 8679436]

46 **Frezza M**, Pozzato G, Chiesa L, Stramentinoli G, di Padova C. Reversal of intrahepatic cholestasis of pregnancy in women after high dose S-adenosyl-L-methionine administration. *Hepatology* 1984; **4**: 274-278 [PMID: 6706301]

47 **Frezza M**, Centini G, Cammareri G, Le Grazie C, Di Padova C. S-adenosylmethionine for the treatment of intrahepatic cholestasis of pregnancy. Results of a controlled clinical trial. *Hepatogastroenterology* 1990; **37** Suppl 2: 122-125 [PMID: 2083923]

48 **Roncaglia N**, Locatelli A, Arreghini A, Assi F, Cameroni I, Pezzullo JC, Ghidini A. A randomised controlled trial of ursodeoxycholic acid and S-adenosyl-l-methionine in the treatment of gestational cholestasis. *BJOG* 2004; **111**: 17-21 [PMID: 14687046]

49 **Kondrackiene J**, Beuers U, Kupcinskas L. Efficacy and safety of ursodeoxycholic acid versus cholestyramine in intrahepatic cholestasis of pregnancy. *Gastroenterology* 2005; **129**: 894-901 [PMID: 16143129 DOI: 10.1053/j.gastro.2005.06.019]

50 **Paumgartner G**, Beuers U. Ursodeoxycholic acid in cholestatic liver disease: mechanisms of action and therapeutic use revisited. *Hepatology* 2002; **36**: 525-531 [PMID: 12198643 DOI: 10.1053/jhep.2002.36088]

51 **Rioseco AJ**, Ivankovic MB, Manzur A, Hamed F, Kato SR, Parer JT, Germain AM. Intrahepatic cholestasis of pregnancy: a retrospective case-control study of perinatal outcome. *Am J Obstet Gynecol* 1994; **170**: 890-895 [PMID: 8141222]

52 **Glantz A**, Marschall HU, Lammert F, Mattsson LA. Intrahepatic cholestasis of pregnancy: a randomized controlled trial comparing dexamethasone and ursodeoxycholic acid. *Hepatology* 2005; **42**: 1399-1405 [PMID: 16317669 DOI: 10.1002/hep.20952]

53 **Glantz A**, Marschall HU, Mattsson LA. Intrahepatic cholestasis of pregnancy: Relationships between bile acid levels and fetal complication rates. *Hepatology* 2004; **40**: 467-474 [PMID: 15368452 DOI: 10.1002/hep.20336]

54 **Rook M**, Vargas J, Caughey A, Bacchetti P, Rosenthal P, Bull L. Fetal outcomes in pregnancies complicated by intrahepatic cholestasis of pregnancy in a Northern California cohort. *PLoS One* 2012; **7**: e28343 [PMID: 22403605 DOI: 10.1371/journal.pone.0028343]

55 Royal College of Obstetricians and Gynecologists. Obstetric cholestasis. London (UK): RCOG Green-top Guideline 2011, No. 43: 7

56 **Joshi D**, James A, Quaglia A, Westbrook RH, Heneghan MA. Liver disease in pregnancy. *Lancet* 2010; **375**: 594-605 [PMID: 20159293 DOI: 10.1016/s0140-6736(09)61495-1]

57 **Kondrackiene J**, Beuers U, Zalinkevicius R, Tauschel HD, Gintautas V, Kupcinskas L. Predictors of premature delivery in patients with intrahepatic cholestasis of pregnancy. *World J Gastroenterol* 2007; **13**: 6226-6230 [PMID: 18069764]

58 **Riely CA**. Acute fatty liver of pregnancy. *Semin Liver Dis* 1987; **7**: 47-54 [PMID: 3296215 DOI: 10.1055/s-2008-1040563]

59 **Pockros PJ**, Peters RL, Reynolds TB. Idiopathic fatty liver of pregnancy: findings in ten cases. *Medicine* (Baltimore) 1984; **63**: 1-11 [PMID: 6690883]

60 **Castro MA**, Goodwin TM, Shaw KJ, Ouzounian JG, McGehee WG. Disseminated intravascular coagulation and antithrombin III depression in acute fatty liver of pregnancy. *Am J Obstet Gynecol* 1996; **174**: 211-216 [PMID: 8572009]

61 **Ibdah JA**. Acute fatty liver of pregnancy: an update on pathogenesis and clinical implications. *World J Gastroenterol* 2006; **12**: 7397-7404 [PMID: 17167825]

62 **Sims HF**, Brackett JC, Powell CK, Treem WR, Hale DE, Bennett MJ, Gibson B, Shapiro S, Strauss AW. The molecular basis of pediatric long chain 3-hydroxyacyl-CoA dehydrogenase deficiency associated with maternal acute fatty liver of pregnancy. *Proc Natl Acad Sci USA* 1995; **92**: 841-845 [PMID: 7846063]

63 **Treem WR**, Shoup ME, Hale DE, Bennett MJ, Rinaldo P, Millington DS, Stanley CA, Riely CA, Hyams JS. Acute fatty liver of pregnancy, hemolysis, elevated liver enzymes, and low platelets syndrome, and long chain 3-hydroxyacyl-coenzyme A dehydrogenase deficiency. *Am J Gastroenterol* 1996; **91**: 2293-2300 [PMID: 8931405]

64 **Treem WR**, Rinaldo P, Hale DE, Stanley CA, Millington DS, Hyams JS, Jackson S, Turnbull DM. Acute fatty liver of pregnancy and long-chain 3-hydroxyacyl-coenzyme A dehydrogenase deficiency. *Hepatology* 1994; **19**: 339-345 [PMID: 8294091]

65 **Isaacs JD**, Sims HF, Powell CK, Bennett MJ, Hale DE, Treem WR, Strauss AW. Maternal acute fatty liver of pregnancy associated with fetal trifunctional protein deficiency: molecular characterization of a novel maternal mutant allele. *Pediatr Res* 1996; **40**: 393-398 [PMID: 8865274 DOI: 10.1203/00006450-199609000-00005]

66 **Yang Z**, Zhao Y, Bennett MJ, Strauss AW, Ibdah JA. Fetal genotypes and pregnancy outcomes in 35 families with mitochondrial trifunctional protein mutations. *Am J Obstet Gynecol* 2002; **187**: 715-720 [PMID: 12237653]

67 **Buytaert IM**, Elewaut GP, Van Kets HE. Early occurrence of acute fatty liver in pregnancy. *Am J Gastroenterol* 1996; **91**: 603-604 [PMID: 8633521]

68 **Monga M**, Katz AR. Acute fatty liver in the second trimester of pregnancy. *Prim Care Update Ob Gyns* 1998; **5**: 191 [PMID: 10838361]

69 **Monga M**, Katz AR. Acute fatty liver in the second trimester. *Obstet Gynecol* 1999; **93**: 811-813 [PMID: 10912403]

70 **Suzuki S**, Watanabe S, Araki T. Acute fatty liver of pregnancy at 23 weeks of gestation. *BJOG* 2001; **108**: 223-224 [PMID:

11236125]
71 **Wilcken B**, Leung KC, Hammond J, Kamath R, Leonard JV. Pregnancy and fetal long-chain 3-hydroxyacyl coenzyme A dehydrogenase deficiency. *Lancet* 1993; **341**: 407-408 [PMID: 8094173]
72 **Mjahed K**, Charra B, Hamoudi D, Noun M, Barrou L. Acute fatty liver of pregnancy. *Arch Gynecol Obstet* 2006; **274**: 349-353 [PMID: 16868757 DOI: 10.1007/s00404-006-0203-6]
73 **Vigil-de Gracia P**, Montufar-Rueda C. Acute fatty liver of pregnancy: diagnosis, treatment, and outcome based on 35 consecutive cases. *J Matern Fetal Neonatal Med* 2011; **24**: 1143-1146 [PMID: 21668324 DOI: 10.3109/14767058.2010.531325]
74 **Holzbach RT**. Acute fatty liver of pregnancy with disseminated intravascular coagulation. *Obstet Gynecol* 1974; **43**: 740-744 [PMID: 4545029]
75 **Burroughs AK**, Seong NH, Dojcinov DM, Scheuer PJ, Sherlock SV. Idiopathic acute fatty liver of pregnancy in 12 patients. *Q J Med* 1982; **51**: 481-497 [PMID: 7156326]
76 **Cano RI**, Delman MR, Pitchumoni CS, Lev R, Rosenthal WS. Acute fatty liver of pregnancy. Complication by disseminated intravascular coagulation. *JAMA* 1975; **231**: 159-161 [PMID: 1172681]
77 **Kueh YK**, Wang TL, Yeo TC, Guan R. A non-fatal case report of acute fatty liver of pregnancy. *Ann Acad Med Singapore* 1991; **20**: 789-791 [PMID: 1803970]
78 **Hsiung R**, Hasselmann M, Lutun P, Gordji M. Acute fatty liver of pregnancy. Diagnostic value of hyperuricemia in the pre-jaundice stage. *J Gynecol Obstet Biol Reprod* (Paris) 1988; **17**: 901-905 [PMID: 3221052]
79 **Knight M**, Nelson-Piercy C, Kurinczuk JJ, Spark P, Brocklehurst P. A prospective national study of acute fatty liver of pregnancy in the UK. *Gut* 2008; **57**: 951-956 [PMID: 18332072 DOI: 10.1136/gut.2008.148676]
80 **Ch'ng CL**, Morgan M, Hainsworth I, Kingham JG. Prospective study of liver dysfunction in pregnancy in Southwest Wales. *Gut* 2002; **51**: 876-880 [PMID: 12427793]
81 **Goel A**, Ramakrishna B, Zachariah U, Ramachandran J, Eapen CE, Kurian G, Chandy G. How accurate are the Swansea criteria to diagnose acute fatty liver of pregnancy in predicting hepatic microvesicular steatosis? *Gut* 2011; **60**: 138-139; author reply 139-140 [PMID: 20938054 DOI: 10.1136/gut.2009.198465]
82 **Minakami H**, Takahashi T, Tamada T. Should routine liver biopsy be done for the definite diagnosis of acute fatty liver of pregnancy? *Am J Obstet Gynecol* 1991; **164**: 1690-1691 [PMID: 1878069]
83 **Seyyed Majidi MR**, Vafaeimanesh J. Plasmapheresis in acute Fatty liver of pregnancy: an effective treatment. *Case Rep Obstet Gynecol* 2013; **2013**: 615975 [PMID: 23424692 DOI: 10.1155/2013/615975]
84 **Dovlatian AA**, Bentsianov VA. Plasmapheresis in the treatment of complicated forms of acute suppurative pyelonephritis in pregnant women. *Urol Nefrol* (Mosk) 1995; (**1**): 22-25 [PMID: 7732626]
85 National High Blood Pressure Education Program Working Group Report on High Blood Pressure in Pregnancy. *Am J Obstet Gynecol* 1990; **163**: 1691-1712 [PMID: 2104525]
86 Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy. *Am J Obstet Gynecol* 2000; **183**: S1-S22 [PMID: 10920346]
87 **Sibai BM**, Mercer B, Sarinoglu C. Severe preeclampsia in the second trimester: recurrence risk and long-term prognosis. *Am J Obstet Gynecol* 1991; **165**: 1408-1412 [PMID: 1957870]
88 **Rolfes DB**, Ishak KG. Liver disease in toxemia of pregnancy. *Am J Gastroenterol* 1986; **81**: 1138-1144 [PMID: 3788923]
89 **Arias F**, Mancilla-Jimenez R. Hepatic fibrinogen deposits in pre-eclampsia. Immunofluorescent evidence. *N Engl J Med* 1976; **295**: 578-582 [PMID: 950973 DOI: 10.1056/nejm197609092951102]
90 **Zhou Y**, McMaster M, Woo K, Janatpour M, Perry J, Karpanen T, Alitalo K, Damsky C, Fisher SJ. Vascular endothelial growth factor ligands and receptors that regulate human cytotrophoblast survival are dysregulated in severe preeclampsia and hemolysis, elevated liver enzymes, and low platelets syndrome. *Am J Pathol* 2002; **160**: 1405-1423 [PMID: 11943725 DOI: 10.1016/s0002-9440(10)62567-9]
91 **Dekker GA**, Robillard PY, Hulsey TC. Immune maladaptation in the etiology of preeclampsia: a review of corroborative epidemiologic studies. *Obstet Gynecol Surv* 1998; **53**: 377-382 [PMID: 9618714]
92 **Pollitt RJ**. Disorders of mitochondrial long-chain fatty acid oxidation. *J Inherit Metab Dis* 1995; **18**: 473-490 [PMID: 7494405]
93 **Barton JR**, Sibai BM. Hepatic imaging in HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet count). *Am J Obstet Gynecol* 1996; **174**: 1820-1825; discussion 1825-1827 [PMID: 8678146]
94 **Barton JR**, Sibai BM. Gastrointestinal complications of preeclampsia. *Semin Perinatol* 2009; **33**: 179-188 [PMID: 19464509 DOI: 10.1053/j.semperi.2009.02.006]
95 **Sibai BM**, Ramadan MK, Usta I, Salama M, Mercer BM, Friedman SA. Maternal morbidity and mortality in 442 pregnancies with hemolysis, elevated liver enzymes, and low platelets (HELLP syndrome). *Am J Obstet Gynecol* 1993; **169**: 1000-1006 [PMID: 8238109]
96 **Sibai BM**. Diagnosis, controversies, and management of the syndrome of hemolysis, elevated liver enzymes, and low platelet count. *Obstet Gynecol* 2004; **103**: 981-991 [PMID: 15121574 DOI: 10.1097/01.aog.0000126245.35811.2a]
97 **Haddad B**, Barton JR, Livingston JC, Chahine R, Sibai BM. Risk factors for adverse maternal outcomes among women with HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome. *Am J Obstet Gynecol* 2000; **183**: 444-448 [PMID: 10942484 DOI: 10.1067/mob.2000.105915]
98 **MacKenna J**, Dover NL, Brame RG. Preeclampsia associated with hemolysis, elevated liver enzymes, and low platelets--an obstetric emergency? *Obstet Gynecol* 1983; **62**: 751-754 [PMID: 6634002]
99 **Heller CS**, Elliott JP. High-order multiple pregnancies complicated by HELLP syndrome. A report of four cases with corticosteroid therapy to prolong gestation. *J Reprod Med* 1997; **42**: 743-746 [PMID: 9408876]
100 **Martin JN**, Blake PG, Perry KG, McCaul JF, Hess LW, Martin RW. The natural history of HELLP syndrome: patterns of disease progression and regression. *Am J Obstet Gynecol* 1991; **164**: 1500-1509; discussion 1509-1513 [PMID: 2048596]
101 Effect of corticosteroids for fetal maturation on perinatal outcomes. NIH Consensus Development Panel on the Effect of Corticosteroids for Fetal Maturation on Perinatal Outcomes. *JAMA* 1995; **273**: 413-418 [PMID: 7823388]
102 **Heyborne KD**, Burke MS, Porreco RP. Prolongation of premature gestation in women with hemolysis, elevated liver enzymes and low platelets. A report of five cases. *J Reprod Med* 1990; **35**: 53-57 [PMID: 2299613]
103 **Tompkins MJ**, Thiagarajah S. HELLP (hemolysis, elevated liver enzymes, and low platelet count) syndrome: the benefit of corticosteroids. *Am J Obstet Gynecol* 1999; **181**: 304-309 [PMID: 10454673]
104 **Varol F**, Aydin T, Gücer F. HELLP syndrome and postpartum corticosteroids. *Int J Gynaecol Obstet* 2001; **73**: 157-159 [PMID: 11336737]

P- Reviewers: Assy N, Iwasaki Y, Manesis EK
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.v19.i43.7647

World J Gastroenterol 2013 November 21; 19(43): 7647-7651
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Liver diseases in pregnancy: Liver transplantation in pregnancy

Ghassan M Hammoud, Ashraf A Almashhrawi, Khulood T Ahmed, Rubayat Rahman, Jamal A Ibdah

Ghassan M Hammoud, Ashraf A Almashhrawi, Khulood T Ahmed, Rubayat Rahman, Jamal A Ibdah, Division of Gastroenterology and Hepatology, University of Missouri, Columbia, MO 65212, United States

Author contributions: Hammoud GM wrote and revised the manuscript; Almashhrawi AA, Ahmed KT and Rahman R were involved in reviewing the literature and collecting data; Ibdah JA provided the overall conceptual design and finalized the manuscript.

Correspondence to: Jamal A Ibdah, MD, PhD, Professor, Director, Division of Gastroenterology and Hepatology, University of Missouri, 319 jesse hall, Columbia, MO 65212, United States. ibdahj@health.missouri.edu

Telephone: +1-573-8827349 Fax: +1-573-8844595
Received: June 17, 2013 Revised: July 30, 2013
Accepted: August 16, 2013
Published online: November 21, 2013

Abstract

Pregnancy in patients with advanced liver disease is uncommon as most women with decompensated cirrhosis are infertile and have high rate of anovulation. However, if gestation ensued; it is very challenging and carries high risks for both the mother and the baby such as higher rates of spontaneous abortion, prematurity, pulmonary hypertension, splenic artery aneurysm rupture, postpartum hemorrhage, and a potential for life-threatening variceal hemorrhage and hepatic decompensation. In contrary, with orthotopic liver transplantation, menstruation resumes and most women of childbearing age are able to conceive, give birth and lead a better quality of life. Women with orthotopic liver transplantation seeking pregnancy should be managed carefully by a team consultation with transplant hepatologist, maternal-fetal medicine specialist and other specialists. Pregnant liver transplant recipients need to stay on immunosuppression medication to prevent allograft rejection. Furthermore, these medications need to be monitored carefully and continued throughout pregnancy to avoid potential adverse effects to mother and baby. Thus delaying pregnancy 1 to 2 years after transplantation minimizes fetal exposure to high doses of immunosuppressants. Pregnant female liver transplant patients have a high rate of cesarean delivery likely due to the high rate of prematurity in this population. Recent reports suggest that with close monitoring and multidisciplinary team approach, most female liver transplant recipient of childbearing age will lead a successful pregnancy.

Key words: Liver; Pregnancy; Liver transplantation; Hemolysis elevated liver low platelets; Acute fatty liver; Cirrhosis

Core tip: This review provides an up-to-date summary of literature in the field of liver transplantation and pregnancy. It outlines the outcomes of pregnancy prior to and after orthotopic liver transplantation. Furthermore, it provides input on preconception counseling for mothers contemplating pregnancy after liver transplantation, risks of immunosuppression, and safety of breastfeeding.

Hammoud GM, Almashhrawi AA, Ahmed KT, Rahman R, Ibdah JA. Liver diseases in pregnancy: Liver transplantation in pregnancy. *World J Gastroenterol* 2013; 19(43): 7647-7651 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7647.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7647

INTRODUCTION

Liver transplantation is considered to be the treatment of choice for patients with advanced liver disease. Since the first pregnancy in a transplant recipient in 1958, pregnancy in recipients of solid organ transplants has become increasingly common. In the setting of decompensated

cirrhosis, pregnancy is very uncommon as most women are infertile and have anovulation and secondary amenorrhea. However, once liver transplantation is performed, liver transplant recipients possess an improved quality of life, their hormonal imbalance return to a normal state, ovulation resumes and pregnancy may ensue if contemplated. The first successful pregnancy in a liver graft recipient was reported in 1978[1]. Given the improving success of liver transplantation over the past two decades and decreasing levels of immunosuppression, most solid organ transplant recipients lead happy and healthy lives with an average 1-year survival rate of greater than 85% for most indications.

PATHOGENESIS

Women with decompensated liver disease commonly have menstrual dysfunction. In fact, menstrual abnormalities may be the first signs of chronic liver disease in females with chronic liver disease. In cirrhotic state, hypothalamic-pituitary dysfunction is associated with an inadequate response to the gonadotropin-releasing hormone agonists and clomiphene citrates as well as diminished gonadotrophin release relative to the reduced levels of circulating sex steroids[2]. Furthermore, serum levels of estradiol and testosterone are increased in patients with portosystemic shunts. Thus pregnancy in decompensated cirrhosis is very uncommon. Obstetrical syndromes associated with transplantation may depend on several factors such as defective deep placentation, underlying maternal diseases, uterine vascular bed and effect of immunosuppressive therapy on uteroplacental arteries[3]. Reports from the the National Transplantation Pregnancy Registry (NTPR) revealed that immunosuppressive medication is associated with an increased risk of miscarriage, prematurity, intrauterine growth retardation, and low birth weight[4].

MATERNAL AND FETAL OUTCOMES IN PREGNANT FEMALE PATIENTS WITH ADVANCED LIVER DISEASE

Pregnancy is associated with increase in portal pressure. During pregnancy, a hypervolemic state develops leading to an increase in portal flow and elevation of portal venous pressure transmitted to the collateral veins with increased risk of esophageal variceal bleeding[5,6]. The outcome of pregnancy in 339 patients with cirrhosis was reported in a large population-based study from 1993 to 2005[7]. Maternal and fetal mortality were much higher than the general population (1.8% *vs* 0%, $P < 0.0001$; 5.2% *vs* 2.1%, $P < 0.0001$) respectively. The rate of hepatic decompensation occurred in 15% and patients with cirrhosis were more likely to deliver by cesarean delivery (42% *vs* 28%; adjusted OR = 1.41; 95%CI: 1.06-1.88). Similarly, the spontaneous abortion rate in cirrhotic patients is approximately 15%-20%.

MATERNAL AND FETAL OUTCOMES IN PREGNANT FEMALE LIVER TRANSPLANT RECIPIENTS

Most outcome data on pregnancy during and after liver transplantation are obtained from the NTPR. The NTPR was established in 1991 at Thomas Jefferson University in Philadelphia, Pennsylvania, to study the outcomes of pregnancies in transplant recipients in North America, including female transplant recipients and those fathered by male transplant recipients. Since then many other reports and case series have been reported and published. A retrospective study from a single institution evaluated a total of 115 gestations in 37 women with liver transplant (LT) and in 34 women with kidney transplant. The authors found 81 (70%) of all gestations were successful, 15 (13%) were terminated, and there were 19 (17%) spontaneous abortions and 2 (2%) intrauterine deaths[8]. Deshpande *et al*[9] reported in a systematic review and meta-analysis outcome of 450 pregnancies in 306 LT recipients in comparisons with the general United States population as well as kidney transplant recipients. The post-LT live birth rate was higher than the live birth rate for the US general population (76.9% *vs* 66.7%, 95%CI: 72.7%-80.7%). The post-LT miscarriage rate was lower than the miscarriage rate for the general population (15.6% *vs* 17.1%, 95%CI: 12.3%-19.2%). Moreover, these rates were similar to the post-kidney transplant rates. The rates of pre-eclampsia, cesarean section delivery and preterm delivery were higher than the rates for the US general population (21.9% *vs* 3.8%, 95%CI: 17.7%-26.4%; 44.6% *vs* 31.9%, 95%CI: 39.2%-50.1%; and 39.4% *vs* 12.5%, 95%CI: 33.1%-46.0%) respectively. Moreover, these rates were lower than those for post-kidney transplant recipients. The overall mean birth weight for newborns of LT recipients was less than the birth weight for the United States general population (2866 g *vs* 3298 g). More notably, the authors found that the mean gestational age and mean birth weight seems significantly greater for liver transplant versus kidney transplant recipients and the risk of hypertension during pregnancy seems also lower for liver transplant than kidney transplant recipients[9]. In another recently published study by Alvaro *et al*[10] from a single center in Spain, the authors analyzed the impact of pregnancy among 1341 liver transplant recipients from April 1986 to April 2011. Thirty pregnancies commenced among 18 liver transplant recipients during the follow-up. Sixteen patients (88%) became pregnant beyond a year after orthotopic liver transplantation. The post-LT live birth was 66.6% and the post-LT abortions were 26.6%. There were no maternal deaths encountered during pregnancy or the postpartum period. However, fetal deaths were observed in 6% of LT recipients. The most common maternal complications during pregnancy were preeclampsia (15%), viral reactivation (15%), acute rejection episodes (10%), infections (10%), and high blood pressure (5%)[10]. Table 1 shows a summary of maternal and fetal outcomes in female liver transplant recipients from selected reports and studies[11].

Table 1 Summary of important fetal and maternal outcomes in liver transplant recipients from selected publications

Author, reference, number of pregnancies	Live birth rate (%)	Preterm (%)	Graft dysfunction (%)	Cesarean section rate (%)	Spontaneous abortions (%)	Low birth weight < 2500 g (%)	Maternal/neonatal deaths (%)
Nagy *et al*[12], *n* = 38	63	29	17	46	NA	17	17/0
Jain *et al*[13], *n* = 49	100	4	25	47	0	9	10/6
Armenti *et al*[14], *n* = 205	73	35	7	35	19	34	/0
Christopher *et al*[15], *n* = 71	71	NA	17	40	19	20	4/0
Dei Malatesta *et al*[16], *n* = 285	78	31	10	43	NA	23	/4
Sibanda *et al*[17], *n* = 16	69	50	NA	62	13	57	NA
Coffin *et al*[18], *n* = 206	70	27	5	38	5	NA	0/6
Jabiry-Zieniewicz *et al*[19], *n* = 39	100	31	8	80	0	20	/0
Dashpande *et al*[9], *n* = 450	76.9	39.4	NA	44.6	6.2 (including intrauterine fetal death)	NA	NA
Alvaro *et al*[10], *n* = 30	66.6	NA	10	42	26.6	NA	0/6

NA: Not available.

Table 2 Food and drug administration pregnancy categories of common immunosuppressive therapy

Medicine	Pregnancy category
Corticosteroids	B
Azathioprine	D
Cyclosporin	C
Mycophenolate mofetil	D
Tacrolimus	C
Sirolimus	C

PRECONCEPTION COUNSELING

Pregnancy after liver transplant should be considered as a high-risk pregnancy and should be monitored closely by a team of a transplant hepatologist and experts in obstetrics and fetal medicine. Female liver transplant recipients who are planning of becoming pregnant should be counseled on optimal timing of pregnancy, mode of delivery and risks associated with immunosuppressive therapy. Furthermore, they should also be counseled on methods of contraception if pregnancy is not contemplated. Immunosuppressive agents are at their nadir one-year post liver transplantation and thus risk of allograft rejection is low. Furthermore, renal and liver functions tend to stabilized during that period and thus it is ideal to delay pregnancy till patient is on a maintenance immunosuppression 1 to 2 years after transplantation to minimizes fetal exposure to high doses of immunosuppressants[14,20].

As per mode of delivery, vaginal delivery appears to be safe. However, high rates of cesarean section have been reported in female liver transplant patients (45.8%[12], 71%[21], 35%[22], 38%[18] and 44.6%[9]) signifying the high rates of prematurity in this population. It is not known whether a particular immunosuppressive therapy is associated with increased rate of cesarean section. If pregnancy is not contemplated in young females of childbearing age, contraceptive method is advised particularly in the first few months post liver transplantation. Barrier methods possess low risk of systemic side effects. Intrauterine devices are generally discouraged due to their potential infection complications. Furthermore, oral contraceptives did not appear to impair liver function or glucose metabolism after its introduction within 6 mo to 7 years post transplantation[23].

IMMUNOSUPPRESSION IN LIVER TRANSPLANT PREGNANT RECIPIENTS

There is no consensus on the optimal maintenance regimen for transplant recipients. The use of immunosuppressive therapy after liver transplantation is unavoidable. Therefore, women planning to conceive after transplantation should be counseled about the risks such therapy may pose on them and their fetuses. All immunosuppressive medication cross the placenta and enter fetal circulation and could potentially have deleterious effects in utero. Despite the fact that immunosuppressive agents such as Azathioprine, Cyclosporine, and Mycophenolic acid, were teratogenic in animals, the risk of birth defects was not statistically different between those who received immunosuppressive medications and those who did not. Birth defects have been reported with Calcineurin inhibitors[8,12,19]. Renal dysfunction and rates of preeclampsia appears higher with cyclosporine therapy[12,24]. No matter what immunosuppressive therapy is chosen based on maternal allograft function and laboratory assay, patients treated with either calcineurin inhibitors cyclosporine or tacrolimus should have serial blood tests in pregnancy to follow medication levels and to assess hepatic and renal function while avoiding unnecessary toxicity. Table 2 shows the food and drug administration classification of risk of medication and their categories in pregnancy[25].

BREASTFEEDING IN FEMALE LIVER TRANSPLANT RECIPIENTS ON IMMUNOSUPPRESSIVE THERAPY

The American academy of pediatrics advises that breast-

feeding mothers can use prednisone and other glucocorticoids safely. Infant exposure to tacrolimus in milk is very low and that maternal tacrolimus therapy may be compatible with breastfeeding. Data collected from the NTPR indicated no adverse outcomes in infants who were breastfed during maternal cyclosporine use. There is insufficient evidence in the literature to suggest that women taking azathioprine should refrain from breastfeeding. Nevertheless, mothers may be discourage to breast feed in the first few months post transplantation where immunosuppressive therapy is at high serum level.

CONCLUSION

Pregnancy after liver transplantation, although considered a high risk pregnancy, has an acceptable outcome for both mother and baby. With the return of fertility following transplantation, accurate family planning advice is essential. To date there is no evidence of specific structural malformations among children born to female liver transplant recipients, but there appears to be increased risk of prematurity and low birth weight after solid organ transplantation. Multidisciplinary team approach is of utmost importance to ensure smooth pregnancy. The NTPR data and others have revolutionized our understanding of the outcomes of pregnancy in this high risk population. We encourage physicians in the field to continue to report their outcome to the transplant registry.

REFERENCES

1 **Walcott WO**, Derick DE, Jolley JJ, Snyder DL. Successful pregnancy in a liver transplant patient. *Am J Obstet Gynecol* 1978; **132**: 340-341 [PMID: 360844]

2 **Van Thiel DH**, Lester R. The effect of chronic alcohol abuse on sexual function. *Clin Endocrinol Metab* 1979; **8**: 499-510 [PMID: 389488 DOI: 10.1016/S0300-595X(79)80028-6]

3 **Brosens I**, Pijnenborg R, Benagiano G. Risk of obstetrical complications in organ transplant recipient pregnancies. *Transplantation* 2013; **96**: 227-233 [PMID: 23466636 DOI: 10.1097/TP.0b013e318289216e]

4 **Coscia LA**, Constantinescu S, Moritz MJ, Frank AM, Ramirez CB, Maley WR, Doria C, McGrory CH, Armenti VT. Report from the National Transplantation Pregnancy Registry (NTPR): outcomes of pregnancy after transplantation. *Clin Transpl* 2010; 65-85 [PMID: 21698831]

5 **d'Alteroche L**, Perarnau JM, Perrotin F, Bacq Y. Portal hypertension and pregnancy. *Gastroenterol Clin Biol* 2008; **32**: 541-546 [PMID: 18472375 DOI: 10.1016/j.gcb.2008.02.028]

6 **Britton RC**. Pregnancy and esophageal varices. *Am J Surg* 1982; **143**: 421-425 [PMID: 6978619 DOI: 10.1016/0002-9610(82)90190-8]

7 **Shaheen AA**, Myers RP. The outcomes of pregnancy in patients with cirrhosis: a population-based study. *Liver Int* 2010; **30**: 275-283 [PMID: 19874491 DOI: 10.1111/j.1478-3231.2009.02153.x]

8 **Blume C**, Sensoy A, Gross MM, Guenter HH, Haller H, Manns MP, Schwarz A, Lehner F, Klempnauer J, Pischke S, Strassburg CP. A comparison of the outcome of pregnancies after liver and kidney transplantation. *Transplantation* 2013; **95**: 222-227 [PMID: 23222883 DOI: 10.1097/TP.0b013e318277e318]

9 **Deshpande NA**, James NT, Kucirka LM, Boyarsky BJ, Garonzik-Wang JM, Cameron AM, Singer AL, Dagher NN, Segev DL. Pregnancy outcomes of liver transplant recipients: a systematic review and meta-analysis. *Liver Transpl* 2012; **18**: 621-629 [PMID: 22344967 DOI: 10.1002/lt.23416]

10 **Alvaro E**, Jimenez LC, Palomo I, Manrique A, Alegre C, Garcia M, Justo I, Abradelo M, Calvo J, Garcia-Sesma A, Cambra F, Loinaz C, Moreno E. Pregnancy and orthotopic liver transplantation. *Transplant Proc* 2013; **45**: 1966-1968 [PMID: 23769084 DOI: 10.1016/j.transproceed.2013.01.013]

11 **Hammoud GM**, Choudhary A, Ibdah JA. Pregnancy and liver transplantation. In: Ibdah JA: Maternal liver disease. Austin (TX): Landes Bioscience, 2012: 119-136

12 **Nagy S**, Bush MC, Berkowitz R, Fishbein TM, Gomez-Lobo V. Pregnancy outcome in liver transplant recipients. *Obstet Gynecol* 2003; **102**: 121-128 [PMID: 12850617 DOI: 10.1016/S0029-7844(03)00369-7]

13 **Jain AB**, Reyes J, Marcos A, Mazariegos G, Eghtesad B, Fontes PA, Cacciarelli TV, Marsh JW, de Vera ME, Rafail A, Starzl TE, Fung JJ. Pregnancy after liver transplantation with tacrolimus immunosuppression: a single center's experience update at 13 years. *Transplantation* 2003; **76**: 827-832 [PMID: 14501862 DOI: 10.1097/01.TP.0000084823.89528.89]

14 **Armenti VT**, Daller JA, Constantinescu S, Silva P, Radomski JS, Moritz MJ, Gaughan WJ, McGrory CH, Coscia LA. Report from the National Transplantation Pregnancy Registry: outcomes of pregnancy after transplantation. *Clin Transpl* 2006; 57-70 [PMID: 18368705]

15 **Christopher V**, Al-Chalabi T, Richardson PD, Muiesan P, Rela M, Heaton ND, O'Grady JG, Heneghan MA. Pregnancy outcome after liver transplantation: a single-center experience of 71 pregnancies in 45 recipients. *Liver Transpl* 2006; **12**: 1138-1143 [PMID: 16799943 DOI: 10.1002/lt.20810]

16 **Dei Malatesta MF**, Rossi M, Rocca B, Iappelli M, Giorno MP, Berloco P, Cortesini R. Pregnancy after liver transplantation: report of 8 new cases and review of the literature. *Transpl Immunol* 2006; **15**: 297-302 [PMID: 16635752 DOI: 10.1016/j.trim.2006.01.001]

17 **Sibanda N**, Briggs JD, Davison JM, Johnson RJ, Rudge CJ. Pregnancy after organ transplantation: a report from the UK Transplant pregnancy registry. *Transplantation* 2007; **83**: 1301-1307 [PMID: 17519778 DOI: 10.1097/01.tp.0000263357.44975.d0]

18 **Coffin CS**, Shaheen AA, Burak KW, Myers RP. Pregnancy outcomes among liver transplant recipients in the United States: a nationwide case-control analysis. *Liver Transpl* 2010; **16**: 56-63 [PMID: 20035524 DOI: 10.1002/lt.21906]

19 **Jabiry-Zieniewicz Z**, Szpotanska-Sikorska M, Pietrzak B, Kociszewska-Najman B, Foroncewicz B, Mucha K, Zieniewicz K, Krawczyk M, Wielgos M. Pregnancy outcomes among female recipients after liver transplantation: further experience. *Transplant Proc* 2011; **43**: 3043-3047 [PMID: 21996220 DOI: 10.1016/j.transproceed.2011.08.070]

20 **Mastrobattista JM**, Katz AR. Pregnancy after organ transplant. *Obstet Gynecol Clin North Am* 2004; **31**: 415-428, vii [PMID: 15200970 DOI: 10.1016/j.ogc.2004.03.005]

21 **Jabiry-Zieniewicz Z**, Bobrowska K, Pietrzak B, Kaminski P, Wielgos M, Durlik M, Zieniewicz K. Mode of delivery in women after liver transplantation. *Transplant Proc* 2007; **39**: 2796-2799 [PMID: 18021990 DOI: 10.1016/j.transproceed.2007.09.011]

22 **Armenti VT**, Constantinescu S, Moritz MJ, Davison JM. Pregnancy after transplantation. *Transplant Rev* (Orlando) 2008; **22**: 223-240 [PMID: 18693108 DOI: 10.1016/j.trre.2008.05.001]

23 **Jabiry-Zieniewicz Z**, Bobrowska K, Kaminski P, Wielgos M, Zieniewicz K, Krawczyk M. Low-dose hormonal contraception after liver transplantation. *Transplant Proc* 2007; **39**: 1530-1532 [PMID: 17580181 DOI: 10.1016/j.transproceed.2007.02.063]

24 **Coscia LA**, Constantinescu S, Moritz MJ, Frank A, Ramirez CB, Maley WL, Doria C, McGrory CH, Armenti VT. Report from the National Transplantation Pregnancy Registry (NTPR): outcomes of pregnancy after transplantation. *Clin Transpl* 2009; 103-122 [PMID: 20524279]

25 **Hammoud GM**, Ibdah JA. The liver in pregnancy. In: Boyer, TD, Manns MP, Sanyal AJ. Zakim and Boyer's Hepatology: A Textbook of Liver Disease. Philadelphia (PA): Elsevier Saunders, 2012: 919-940 [DOI: 10.1016/B978-1-4377-0881-3.00052-8]

P- Reviewers: Muratori L, Yamagiwa S **S- Editor:** Wen LL
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.v19.i43.7652

World J Gastroenterol 2013 November 21; 19(43): 7652-7660
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

Use of exclusive enteral nutrition in adults with Crohn's disease: A review

Catherine L Wall, Andrew S Day, Richard B Gearry

Catherine L Wall, Andrew S Day, Department of Paediatrics, University of Otago, Christchurch 8140, New Zealand
Richard B Gearry, Department of Medicine, University of Otago, Christchurch 8140, New Zealand
Author contributions: Wall CL collected and collated the data and drafted the manuscript; Day AS and Gearry RB conceived the article idea, participated in its design and contributed to draft manuscripts; All authors read and approved the final manuscript.
Correspondence to: Andrew S Day, Professor, Department of Paediatrics, University of Otago, Christchurch, 2 Riccarton Ave, PO Box 4345, Christchurch 8140, New Zealand. andrew.day@otago.ac.nz
Telephone: +64-3-3640747 Fax: +64-3-3786355
Received: May 24, 2013 Revised: July 20, 2013
Accepted: September 16, 2013
Published online: November 21, 2013

Abstract

Exclusive enteral nutrition (EEN) is well-established as a first line therapy instead of corticosteroid (CS) therapy to treat active Crohn's disease (CD) in children. It also has been shown to have benefits over and above induction of disease remission in paediatric populations. However, other than in Japanese populations, this intervention is not routinely utilised in adults. To investigate potential reasons for variation in response between adult studies of EEN and CS therapy. The Ovid database was searched over a 6-mo period. Articles directly comparing EEN and CS therapy in adults were included. Eleven articles were identified. EEN therapy remission rates varied considerably. Poor compliance with EEN therapy due to unpalatable formula was an issue in half of the studies. Remission rates of studies that only included patients with previously untreated/new CD were higher than studies including patients with both existing and new disease. There was limited evidence to determine if disease location, duration of disease or age of diagnosis affected EEN therapy outcomes. There is some evidence to support the use of EEN as a treatment option for a select group of adults, namely those motivated to adhere to an EEN regimen and possibly those newly diagnosed with CD. In addition, the use of more palatable formulas could improve treatment compliance.

Key words: Exclusive enteral nutrition; Crohn's disease; Adults

Core tip: Exclusive enteral nutrition (EEN) is an established treatment for children with active Crohn's disease (CD). At present, this therapy is used sparingly in adult patients outside of Japan. In reviewing the published literature regarding the use of EEN in adult patients, this article highlights evidence supporting the use of EEN as a treatment option for selected patients: namely those motivated to adhere to an EEN regimen and those newly diagnosed with CD. The role of EEN in adult patients with CD should now be re-examined, with particular regard to treatment protocols and the use of more palatable polymeric formulae that may enhance compliance.

Wall CL, Day AS, Gearry RB. Use of exclusive enteral nutrition in adults with Crohn's disease: A review. *World J Gastroenterol* 2013; 19(43): 7652-7660 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7652.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7652

INTRODUCTION

Crohn's disease (CD) is an incurable inflammatory bowel disease (IBD) characterised by inflammation of the gastrointestinal tract, which leads to chronic symptoms such as diarrhoea, abdominal pain and rectal bleeding[1]. The peak age of diagnosis is between 15 and 30 years of age,

leading to many years of disease and associated morbidity. Standard first line treatment in adults newly diagnosed with CD is corticosteroid (CS) therapy, which is effective at inducing remission or response in approximately 85% of patients[2]. However, CS therapy has many well documented acute side-effects: furthermore there are numerous long term adverse effects due to repeated or continual use of CS[3]. Also, CS resistance can occur in 8%-22% of patients and CS dependency occurs in 15%-36% of patients[4]. Alternative therapies that can effectively induce and maintain disease remission without short and long term side effects are desirable.

Exclusive enteral nutrition (EEN) is the provision of 100% of a person's nutritional requirements from a liquid nutrition formula either orally or *via* a feeding tube. EEN is usually provided for 6-8 wk and then usual diet is gradually reintroduced[5]. In children with CD, EEN has been shown to be an effective and feasible alternative to CS[6]. In addition to avoiding the adverse effects of CS exposure, EEN provides additional benefits over and above those provided by CS. EEN therapy is associated with higher rates of mucosal healing[7], altered intestinal flora[8], greater weight gain[9], improved vitamin D status[10], enhanced bone turnover[11], an early rise in Insulin-like growth factor 1[12], and better quality of life after treatment[13]. There are few long term follow up studies post EEN, but those that have been conducted in children indicate that EEN may improve time to relapse[14]. The administration of supplementary enteral nutrition (SEN) once disease remission is achieved has been shown to be beneficial in maintaining remission compared with a free diet in Japanese adults[15] and children[16].

However, in adult CD populations, EEN is generally not seen as a first line therapy for newly diagnosed or those with a flare of pre-existing CD. European[2] and North American[17] clinical guidelines only recommend EEN if a patient declines drug therapy or as an adjunctive therapy to support nutrition, rather than as a primary therapy. These recommendations are primarily based on the results of a Cochrane systematic review of six randomised controlled trials including 192 patients treated with EEN and 160 patients treated with CS[18]. The review found a pooled OR of 0.33 (95%CI: 0.21-0.53) in favour of CS and concluded that CS were superior to EEN in the induction of remission of disease. In contrast to these guidelines, recent Japanese experience demonstrates efficacy in that setting[15].

It is not clear why the benefits of EEN therapy seen in paediatric populations are not achieved in adults. We aimed to review the published literature reporting the use of EEN as a primary therapy for active CD in adults and examine potential reasons for this apparent discrepancy.

SEARCH

The Ovid database was searched from September 2012 to March 2013 for articles published between 1946 and now. Key search terms were: "CD", "Crohn disease", "EEN" and "enteral nutrition". Abstracts were scanned and articles in English that compared enteral nutrition with CS treatment in adults were considered relevant. Studies were excluded if enteral nutrition was not the sole source of nutrition, enteral nutrition was provided as well as other medication (for example, antibiotics), the study included children, or the study did not compare CS and EEN. A manual search was also completed of reference lists of articles retrieved, relevant review articles and meta-analyses on the topic.

RESEARCH

Study characteristics

Eleven studies published between 1984 and 2002 were identified that compared EEN with CS treatment in adults (Table 1). Two were abstracts[19,20] and the rest were full articles. The studies were conducted in Europe, North America and Asia: three in England[19,21,22], one in Spain[23], one in Greece[20], one in Italy[24], one in the United States[25], one in Japan[26] and three[27-29] were multicentre European trials. All but two studies enrolled a mix of patients with newly diagnosed CD (naïve to prior treatment) and existing CD. All but one study compared one enteral nutrition formula with CS therapy.

The studies utilised a range of nutritional products, in varying regimens, as summarised in Table 2. Eight of the studies used elemental formula and three studies used polymeric formula. Most formulas were a 1 kcal/mL concentration apart from one which used a 1.5 kcal/mL formula. Duration of EEN treatment ranged from 2-6 weeks but most studies used EEN therapy for four weeks. Mode of delivery of the EN formula was either orally, or *via* a nasogastric tube (NGT) if not tolerated orally, or continuous feeding *via* an NGT or nasoduodenal tube. Nutritional composition of the formulas was quite different depending on the type and brand of formula used. All formulas had relatively similar amounts of protein (14%-22% of total energy), whereas fat content varied considerably (1%-35% of total energy). Carbohydrate content varied relative to fat content (49%-82% of total energy).

The only study that compared two different enteral formulas and CS was published by Gassull *et al*[27] They compared two EEN formulas that were the same except for the predominant type of fat: one was high in oleic acid and the other was high in linoleic acid. Study recruitment was ended prematurely because less than 33% of the high oleic acid formula group had achieved disease remission and the remission rate was significantly different from that of the other treatments.

CS protocols also ranged between the evaluated studies. Usual initial CS dosage was between 0.5 mg/kg per day and 1.0 mg/kg per day, with subsequent weaning courses. CS were given orally in two studies[23,27] but the route of administration was not published in the majority of studies. Two studies administered CS and sulfasalazine concurrently[28,29].

Table 1 Studies of adults that compared exclusive enteral nutrition with corticosteroid therapy

Ref.	Year	Country	Age (SD or range)	Received no previous CD treatment (% of EEN group)	Number of participants		% that achieved remission (intention to treat)		Significant difference (*P* value)	Remission criteria	Number that did not complete EEN intervention, *n* (%)		% that achieved remission (treatment completed)	
					EEN	CS	EEN	CS			Formula un-palatable	Other reason	EEN	CS
Engelman *et al*[19]	1993	England	23-54	Not stated	7	4	100%	100%	NS	HBI < 6.0	0 (0)	0	100%	100%
Gassull *et al*[27]1	2002	Europe	31.3 (3.3)	50%	20	19	20%	79%	0.0005	VHAI < 120	5 (25)	0	27%	79%
Gassull *et al*[27]2	2002	Europe	30.8 (4.1)	43.50%	23	19	52%	79%	NS	VHAI < 120	4 (17)	0	63%	79%
González-Huix *et al*[23]	1993	Spain	31.1 (4.1)	47%	15	17	8%	88%	NS	VHAI < 120	0 (0)	0	80%	88%
Gorard *et al*[21]	1993	England	31.6 (3.0)	50%	22	20	45%	85%	< 0.05	HBI - remission not defined , mean < 2	9 (41)	2	91%	89%
Lindor *et al*[25]	1992	United States	34.7 (26-64)	33%	9	10	50%	33%	NS	CDAI decrease > 100 points	3 (33)	1	60%	63%
Lochs *et al*[28]	1991	Europe	27.5 (1.5)	Not stated	55	52	53%	79%	< 0.01	CDAI decrease > 100 points or > 40%	7 (13)	0	60%	85%
Malchow *et al*[29]	1990	Europe	30.1 (11.5)	20%	51	44	41%	71%	< 0.05	CDAI decrease > 100 points or > 40%	20 (39)	0	71%	91%
Mantzaris *et al*[20]	1996	Greece	Not stated	20%	10	10	40%	70%	NS	CDAI < 150 or decrease > 100 points	0 (0)	0	40%	70%
Okada *et al*[26]	1990	Japan	21.0 (3.3)	100%	10	10	80%	30%	< 0.01	HBI < 1	0 (0)	0	80%	30%
O'Moráin *et al*[22]	1984	England	31.9 (15-60)	100%	11	10	82%	80%	NS	HBI - remission not defined. Mean < 3	2 (18)	0	100%	100%
Zoli *et al*[24]	1997	Italy	33.5 (15.9)	Not stated	12	10	67%	50%	NS	HBI < 3	1 (8)	1	80%	50%

[1,2]Gassull *et al* had two EEN arms: [1]High oleic fatty acid formula; [2]High linoleic fatty acid formula. CD: Crohn's disease; CDAI: Crohn's disease activity index; CS: Corticosteroids; EEN: Exclusive enteral nutrition; HBI: Harvey Bradshaw Index; NS: Non-significant; VHAI: Van Hees activity index.

Disease remission criteria

Three remission criteria were used across the 11 studies - the CD Activity Index (CDAI), the Harvey Bradshaw Index (HBI), and the Van Hees Activity Index (VHAI). The CDAI score uses a seven day history of general well-being, abdominal pain, loose stools, presence of abdominal mass and CD complications, anti-diarrhoeal use, haematocrit and weight[30]. The HBI is based on a one day history of general well-being, abdominal pain, loose stools and presence of abdominal mass and CD complications[30]. It correlates well with the CDAI ($r = 0.8$)[30]. Clinical remission is usually defined as a CDAI of less than or equal to 150 points or a HBI of less than or equal to 4 points[30]. Of the four studies that used the CDAI to define remission one used this criteria, one used a decrease of more than 100 points and two used either a CDAI of less than 150 or a decrease of 40% or more. Five studies used the HBI to define disease remission, the cut-offs used by each study were different.

The VHAI is calculated using serum albumin and erythrocyte sedimentation rate, body mass index, abdominal mass, gender, fever, loose stools, bowel resection and CD complications. The VHAI correlates moderately ($r = 0.67$) with the CDAI[31]. Both studies that used the VHAI used the same cut-off of less than 120 to define disease remission.

Remission of disease

Remission was achieved with EEN therapy on an intention to treat basis in 20%-100% of patients and 30%-100% of patient on CS therapy (Table 1). Seven of the 11 studies found no significant difference between EEN and CS treatment to induce disease remission[19,20,22-25,27]. Of those patients who completed the course of EEN therapy disease remission was achieved in 23%-100% of patients and in 30%-100% of patients that completed CS treatment[19-29]. Those that did not complete the course of EEN therapy were

Table 2 Characteristics of exclusive enteral nutrition regimens used in studies of adults that compared exclusive enteral nutrition with corticosteroid therapy

Ref.	Nutritional product	Type of feed	Duration of EEN (wk)	Calorie density (kcal/mL)	Nutritional composition (% TE)	Mode of delivery	Calorie intake per day
Engelman *et al*[19]	Peptamen	Peptide based elemental	2	1	Pro 16, CHO 51, Fat 33	Orally	30-35 kcal/kg per day
Gassull *et al*[27]	High oleic acid	Polymeric (powder)	4	1	Pro 22, CHO 46, Fat 32	Orally and NGT	Not stated
Gassull *et al*[27]	High linoleic acid	Polymeric (powder)	4	1	Pro 22, CHO 46, Fat 32	Orally and NGT	Not stated
González-Huix *et al*[23]	Edanec HN	Polymeric	4	1	Pro 22, CHO 46, Fat 32	NGT	Not stated
Gorard *et al*[21]	Vivonex TEN	Elemental	4	1	Pro 15, CHO 82, Fat 3	Orally, or NGT	2100 kcal per day
Lindor *et al*[25]	Vital HN	Peptide based elemental	4	1	Pro 17, CHO 74, Fat 9	Orally	40 kcal/kg per day
Lochs *et al*[28]	Peptisorb	Peptide based elemental	4-6	1	Pro 16, CHO 69, Fat 15	NGT or NDT	35 kcal/kg per day
Malchow *et al*[29]	Survimed	Peptide based elemental	3-6	1	Pro 14, CHO 76, Fat 10	Orally	33 kcal/kg per day
Mantzaris *et al*[20]	Nutrison HE	Polymeric	4	1.5	Pro 16, CHO 49, Fat 35	NDT	2250 kcal per day
Okada *et al*[26]	Elental	Elemental	6	1	Pro 19, CHO 81, Fat 1	NDT	40-60 kcal/kg per day
O'Moráin *et al*[22]	Vivonex	Elemental	4	1	Pro 15, CHO 82, Fat 3	Orally, or NGT	40-60 kcal/kg per day
Zoli *et al*[24]	Peptamen	Peptide based elemental	2	1	Pro 16, CHO 51, Fat 33	Orally	Not stated

CHO: Carbohydrate; EEN: Exclusive enteral nutrition; NDT: Nasoduodenal tube; NGT: Nasogastric tube; Pro: Protein; % TE: Percentage of total energy.

usually started on CS therapy.

Withdrawal from treatment

Withdrawals from treatment varied between studies. EEN study group withdrawals were mostly due to unpalatable enteral nutrition formula. The number of withdrawals for this reason was as high as 41% of the EEN group in one study but 0% in other EEN study groups. Occasionally patients had to withdraw as they required urgent surgery. Withdrawals from CS groups were much lower. Common reasons cited for withdrawing were side effects, non-compliance with treatment or the patient needing urgent surgery.

Disease location

All 11 of the studies recorded the disease location of patients. The majority of patients had ileocolonic disease and smaller numbers had ileal or isolated colonic disease. No studies found disease location to be associated with the likelihood of achieving disease remission using EEN or CS therapy.

Age of participants

The age of the participants was recorded differently across the 11 studies. The mean age of patients enrolled in the studies was 27.5-34.7 years old. Inclusion of older adults aged more than 50 years of age was not uncommon. Only one study included mostly younger adults (mean 21.0 ± 3.3 years)[26].

DISCUSSION

EEN is rarely used in adults with active CD, apart from in Japan. Its use is usually reserved for those patients who do not want to use CS therapy, as an adjunctive therapy or where other treatment options have failed. Since the first studies with adults in the 1980s and 1990s much more is known about the way in which EEN therapy induces disease remission in children and how SEN therapy can assist in maintenance of disease remission. It is timely to readdress the possible reasons for the discrepancy between results from adult and paediatric studies that have compared EEN and CS therapy.

Disease remission criteria

The disease remission criteria used by researchers can have a profound impact on the study results. Comparison of disease remission rates between studies is challenging when disease remission is not universally defined. Five of the 11 studies used the HBI to measure disease remission[19,21,22,24,26]. Two of the studies that used the HBI did not describe their remission criteria[21,22]; however the mean HBI of participants after the EEN intervention was less than 4, which corresponds with standard interpretations of clinical remission. Another study used a HBI cut off of less than six points with 100% of participants in both the EEN and CS therapy groups achieving remission in this study[19]. The fourth study to use the HBI used a cut-off of 0-1 points to define disease remission[26]. Only 30% of patients in the CS group achieved remission using this criterion compared with 80% of the EEN group. It is unknown if a more liberal cut-off would have increased the number of patients achieving disease remission in the CS group. Regardless of the HBI cut-off used at least 80% of the EEN group participants (that completed the course of EEN) in each of the five studies achieved disease remission.

Four of the 11 studies used the CDAI to measure dis-

ease remission[20,25,28,29]. The remission rates of the EEN therapy group in all four studies were low (40%-53%), with the two larger studies concluding that, on an intention to treat basis, CS therapy induces disease remission in significantly more patients that EEN therapy[28,29]. In two of the studies at least one third of the patients withdrew from the EEN group due to unpalatable formula[25,29]. Withdrawals from the CS groups were much lower (20% or less). Of those that did complete the course of EEN therapy only 40%-71% of patients achieved disease remission, whereas remission was achieved in 62%-98% of those that completed the course of CS therapy.

The disease remission rates of the two studies that used the VHAI to define disease remission were quite different. Gassull *et al*[27] hypothesised that the formula high in linoleic acid, an n-6 polyunsaturated fat, would be less effective than a high monounsaturated fatty acid formula because n-6 fatty acids are pro-inflammatory precursors. Of the 20 patients enrolled in the high oleic acid EEN group only 20% achieved disease remission after 4 wk of therapy, compared with 52% of the high linoleic acid group and 79% of those using CS therapy. It seems that the fat content of EEN formulae may affect the efficacy of EEN therapy. The other study that used the VHAI to define disease remission found that EEN therapy was as effective as CS therapy: 80% of those on EEN therapy achieved disease remission compared with 88% of those using CS therapy[23].

The criteria used to define disease remission should not impact greatly on the results of the study; however, in this case, the studies can be grouped into three categories based on the remission criteria applied. The studies that used the HBI found that EEN therapy was at least as effective as CS therapy in inducing disease remission. The two larger studies that used the CDAI found that CS therapy was superior to EEN therapy while two studies with small participant numbers found no significant difference. There may be differences in study protocols between studies with higher and lower patient numbers that could influence patient outcomes. Finally, the two studies that used the VHAI found that there was no significant difference between a high, or a moderate, polyunsaturated polymeric formula and CS therapy, but that a high monounsaturated formula was significantly less effective ($P < 0.001$) than CS therapy at inducing disease remission.

Newly diagnosed CD

There is some evidence to suggest that EEN therapy is more effective in newly diagnosed CD patients compared with patients who have existing CD. Differences in treatment response rates according to time since diagnosis are not limited to EEN therapy. Response and remission rates achieved with biologic therapy are greater in children than adults[32] which may, in part, be due to the duration of disease prior to initiation of the treatment. Similarly, adults with a shorter duration of CD are more likely to respond and achieve remission with biologic therapy[32]. Also the use of immune-modulators early in the disease course in adults and children has been shown to reduce the probability of long term CS and intestinal surgeries[33].

Two adult studies have compared EEN with CS therapy in treatment-naïve patients[22,26]. In both studies, 80% of those treated with EEN achieved disease remission after 4-6 wk of an elemental diet (comparable to remission rates in those treated with CS). Other adult studies comparing EEN with CS have not differentiated between patients with newly diagnosed CD and existing CD in their analyses. One study mentioned that both of the newly diagnosed CD patients responded to EEN treatment[20], but the numbers enrolled in the study were too small to show if there was a statistically significant difference in response to treatment between the two groups. A study of 22 patients treated with EEN found that EEN therapy was as effective in newly diagnosed patients as those with existing disease[21], although 40% of patients did not complete the course of EEN. The authors do not indicate how many of those that completed EEN treatment had existing or newly diagnosed disease. The two larger multi-centre European trials did not differentiate between those that had and not had received previous CD treatment[28,29].

Paediatric research suggests that EEN is more effective in treating newly diagnosed CD than existing CD[9]. Day *et al*[9] showed that, of 15 newly diagnosed CD patients, 12 (80%) entered remission after eight weeks of EEN, whereas only seven of the 12 (58%) children with long-standing disease entered remission ($P > 0.05$ by fishers exact test). In other paediatric studies with newly diagnosed CD patients disease remission was achieved in 79%-93% of those that completed EEN treatment and 70%-79% on an intention to treat basis[7,34].

Duration of CD

Longer duration of CD is associated with more complications including tissue scaring, fistulae, abscess, strictures, perianal disease and bowel resections[35]. EEN therapy has been shown to induce disease remission by reducing mucosal inflammation[36-38]. Complications of CD are often non-inflammatory in nature; therefore, EEN may be less effective in treating these patients. Interestingly, a case series of three children with perianal disease at diagnosis found that EEN (used in combination with surgery and antibiotics) was effective at inducing disease remission and assisted in the healing of perianal disease[39]. EEN was used as a maintenance therapy in all three children without return of perianal disease. A clinical trial has not been conducted to further investigate the potential role of EEN in the management of perianal CD.

Overall, studies in adult patients of EEN compared with CS therapy have not excluded patients with complicated disease. Usual exclusions included imminent surgery, intestinal perforation, ileus, abscesses, massive bleeding, short bowel syndrome with ileostomy and, in

some cases, previous surgery. The presence of other complications of existing CD such as scaring, perianal disease or previous bowel surgery is not detailed in the adult literature. It is impossible to ascertain whether those who did not respond to EEN therapy had more or less complications than those who did respond. Furthermore, the studies had only small numbers of patients within each disease sub-group and were unable to conduct in-depth statistical analysis of these sub-groups.

Adherence

Non-adherence with EEN treatment was a limiting factor in the success of EEN therapy in many studies. A number of reasons for non-adherence of adult CD patients with EEN therapy have been postulated including poor taste of the formula, lack of support and poor motivation to complete the treatment.

Un-palatability of the EN formula was the most common reason for non-adherence in the studies performed to date. Many early studies that compared EEN with CS treatment used elemental formulas. The difference between polymeric and elemental formulas is that the protein fraction in polymeric formula is in its whole form rather than as individual amino acids or peptides in semi-elemental formulas and elemental formulas tend to have a low total fat content Polymeric formula has been shown to be as effective as elemental at inducing disease remission[40,41]. Elemental formulas have a distinctive smell and flavour mainly due to the presence of amino acids, which have a bitter flavour. Bitterness is negatively correlated with palatability, whereas sweetness and sourness are positively correlated with palatability[42]. Fat content may also affect the palatability of the formula[43]. The elemental formulas used in the studies were low fat (1%-3% TE) compared with semi-elemental (9%-33% TE) and polymeric (32%-35% TE) formulas. Hence polymeric formulas are thought to be more palatable. However, there is limited research comparing the palatability of the two formula types. A retrospective study of children who received elemental formula from 1992-2001 and children who received polymeric formula from 2000-2004 found that adherence to treatment did not differ between the two groups but that those receiving polymeric formula were less likely to need a nasogastric tubes (NGT) inserted to deliver the feed[44].

The mode of delivery of the formula may also play a role in patient compliance. Many studies with high adherence rates administered elemental formulas *via* NG or nasoduodenal tubes rather than orally. More recent paediatric studies have encouraged oral intake of polymeric formula and use of NGT only if needed[7,9,34]. For free living (non-hospitalised) patients, taking the formula orally may be more socially acceptable. Elemental and polymeric formulas have been shown to be as equally effective at inducing remission of disease in children[40] and adults[18].

Studies that used elemental formulas given exclusively *via* NG or nasoduodenal tubes had low rates of non-adherence (0%-13%)[26,28]. Whereas studies that reported high rates of non-adherence (33%-41%) used elemental or semi-elemental formulas given orally and if a patient did not tolerate EEN orally a NGT was placed.[21,25,29] However, three of the six studies using elemental or semi-elemental diet orally reported higher adherence rates[19,22,24]. Two of these studies[19,24] only used EEN for 2 wk and patients were given a peptide based semi-elemental formula (Peptamen) orally rather than an amino acid-based elemental formula. Of the 19 patients using EEN in these two trials, only 1 patient was non-adherent with the treatment. The third study, by O'Moráin *et al*[22] was one of the first to compare EEN to CS treatment. Patients were asked to take the elemental formula orally for four weeks and if they could not tolerate it a NGT was placed. Of the 11 patients in the EEN group, two (18%) could not tolerate the formula orally or *via* a NGT.

Of the three adult studies that used polymeric formula, two administered it *via* NG or nasoduodenal tubes with 100% adherence[20,23]. The third study used a polymeric powder (a high oleic and high linoleic acid formulation) given orally or *via* NGT if not tolerated orally[27]. Non-adherence with the treatment was 17%-25%. No published adult studies have used a ready-to-drink polymeric formula given orally. There are, however, various studies with children that have shown that polymeric formulas are palatable orally. Borrelli *et al*[7] studied 19 children with CD who drank an isocaloric polymeric formula (Modulen) as their sole source of nutrition for 10 wk. Thirteen children took the formula orally; four required overnight feeding *via* a NGT, in addition to taking it orally during the day, to meet their nutritional requirements and two children could not manage to take the required volume of formula orally or *via* a NGT. Of the 17 children that successfully completed the 10 wk intervention 15 (88%) achieved disease remission. Day *et al*[9] studied 27 children with CD who were prescribed EEN with isocaloric polymeric formula (Modulen or Osmolite) for up to 8 wk. Nineteen children managed the required volume of formula orally, five needed to take some of the formula *via* a NGT and three could not tolerate the required volume orally or *via* a NGT. Of the 24 children who completed at least 8 wk of EEN, 19 entered remission (79%).

Both of these paediatric studies used an isocaloric polymeric formula. It appears that the major reason for non-adherence in these cohorts was difficulty tolerating the volume required for nutritional requirements rather than un-palatability. It is not clear whether the volume required to meet an adult's nutritional requirements (*e.g.*, 1600-2400 mL of ready-to-drink isocaloric polymeric formula per day) may lead to poor adherence. The use of a concentrated polymeric formula, (*e.g.*, 1.5 kcal/mL formula), may help alleviate this issue.

If adherence with and response to EEN treatment of 90% and 80%, respectively, can be achieved in adults EEN may be aviable treatment option. Ready-to-drink polymeric formula, which may be more palatable orally

than elemental or semi-elemental formulas and more convenient and portable than powdered options, could provide an option for adults with CD wishing to reduce their exposure to CS, induce disease remission and potentially attain the benefits associated with EEN therapy that have been confirmed in children.

Disease location

Disease location is thought to affect the efficacy of EEN therapy. In particular, colonic disease may be more refractory to treatment than disease with ileal involvement. However, due to the small participant numbers in most adult EEN studies there has been insufficient statistical power for subgroup analyses. A pooled meta-analysis of mainly adult studies from the 1980s and 1990s found that there was insufficient data to perform subgroup analyses by disease location[18].

Some paediatric studies have specifically investigated the impact of disease location on response to EEN therapy. Afzal *et al*[13] studied 65 children aged 8-17 years old with newly diagnosed CD of which 12 had ileal disease, 39 had ileocolonic disease and 14 had isolated colonic disease. They found that disease remission was harder to induce with EEN therapy in patients with colonic disease - remission achieved in 50% compared with 82% in those with ileocolonic disease and 92% in those with ileal CD ($P = 0.02$). They also used colonoscopy to assess mucosal healing after EEN therapy and found that there was no improvement in colonic mucosal inflammation in those with colonic or ileocolonic disease.

Conversely, Buchman *et al*[3] investigated the effect of disease location on remission rates after EEN therapy and found that colonic CD responded just as well as ileocolonic disease. Their study included 114 children (median age 11.6 years), all with recently diagnosed CD. Nineteen patients had colonic disease, four had ileal disease, 29 had ileocolonic disease, 49 had upper gastrointestinal tract disease and 9 had disease that could be not be classified using the Vienna classification. Of those with colonic disease 79% went into remission after eight weeks of EEN therapy compared with 86% with ileocolonic disease, 88% with upper gastrointestinal disease and only 25% with ileal disease. It should be noted that there were only 4 patients with ileal disease compared with at least 20 in the other three groups. Further evidence is needed to confirm whether CD location affects the efficacy of EEN.

Age of patient

Current guidelines suggest that EEN therapy is more appropriate to use in paediatric rather than adult patients[6,18]. There are no studies in adults that have assessed whether age affects response to EEN therapy. Although the mean age of adults included in the 11 studies evaluated here was approximately 30 years, the age range varied substantially and was not always published. Of those that did publish the age range of patients it was common to include patients aged 20 up to 50 or 60[19,22,25]. It is unknown if age affects response to EEN therapy or compliance with treatment.

CONCLUSION

Initial reports demonstrated that EEN was effective in inducing remission in adults with active CD and proposed this intervention as an alternative to CS therapy. However, subsequent larger studies failed to reproduce these results. Since then many studies have been conducted in paediatric populations and numerous benefits over and above achieving disease remission have become apparent. It appears that non-compliance with EEN treatment in early studies adversely affected the efficacy of EEN compared with CS therapy. There is also evidence to support a possible role of EEN with a specific group of adult patients - those newly diagnosed disease and, possibly, those with ileal involvement. Further research with this group is warranted. The use of polymeric formulas provided orally, which has not previously been studied in adult patients, may improve treatment compliance and allow adult patients to reap the many other benefits of EEN that have been shown in children over and above achieving disease remission and improving nutritional status.

REFERENCES

1 **Baumgart DC**, Sandborn WJ. Crohn's disease. *Lancet* 2012; **380**: 1590-1605 [PMID: 22914295 DOI: 10.1016/s0140-6736(12)60026-9]

2 **Dignass A**, Van Assche G, Lindsay JO, Lémann M, Söderholm J, Colombel JF, Danese S, D'Hoore A, Gassull M, Gomollón F, Hommes DW, Michetti P, O'Morain C, Oresland T, Windsor A, Stange EF, Travis SP. The second European evidence-based Consensus on the diagnosis and management of Crohn's disease: Current management. *J Crohns Colitis* 2010; **4**: 28-62 [PMID: 21122489 DOI: 10.1016/j.crohns.2010.07.001]

3 **Buchman AL**. Side effects of corticosteroid therapy. *J Clin Gastroenterol* 2001; **33**: 289-294 [PMID: 11588541 DOI: 10.1097/00004836-200110000-00006]

4 **Gelbmann CM**, Rogler G, Gross V, Gierend M, Bregenzer N, Andus T, Schölmerich J. Prior bowel resections, perianal disease, and a high initial Crohn's disease activity index are associated with corticosteroid resistance in active Crohn's disease. *Am J Gastroenterol* 2002; **97**: 1438-1445 [PMID: 12094862]

5 **Whitten KE**, Rogers P, Ooi CY, Day AS. International survey of enteral nutrition protocols used in children with Crohn's disease. *J Dig Dis* 2012; **13**: 107-112 [PMID: 22257479 DOI: 10.1111/j.1751-2980.2011.00558.x]

6 **Heuschkel RB**. Enteral nutrition in children with Crohn's disease. *J Pediatr Gastroenterol Nutr* 2000; **31**: 575 [PMID: 11144448 DOI: 10.1097/00005176-200011000-00024]

7 **Borrelli O**, Cordischi L, Cirulli M, Paganelli M, Labalestra V, Uccini S, Russo PM, Cucchiara S. Polymeric diet alone versus corticosteroids in the treatment of active pediatric Crohn's disease: a randomized controlled open-label trial. *Clin Gastroenterol Hepatol* 2006; **4**: 744-753 [PMID: 16682258 DOI: 10.1016/j.cgh.2006.03.010]

8 **Andoh A**, Tsujikawa T, Sasaki M, Mitsuyama K, Suzuki Y, Matsui T, Matsumoto T, Benno Y, Fujiyama Y. Faecal microbiota profile of Crohn's disease determined by terminal restriction fragment length polymorphism analysis. *Aliment Pharmacol Ther* 2009; **29**: 75-82 [PMID: 18945264 DOI: 10.1111/j.1365-2036.2008.03860.x]

9 **Day AS**, Whitten KE, Lemberg DA, Clarkson C, Vitug-Sales M, Jackson R, Bohane TD. Exclusive enteral feeding as primary therapy for Crohn's disease in Australian children and adolescents: a feasible and effective approach. *J Gastroenterol Hepatol* 2006; **21**: 1609-1614 [PMID: 16928225 DOI: 10.1111/j.1440-1746.2006.04294.x]

10 **Levin AD**, Wadhera V, Leach ST, Woodhead HJ, Lemberg DA, Mendoza-Cruz AC, Day AS. Vitamin D deficiency in children with inflammatory bowel disease. *Dig Dis Sci* 2011; **56**: 830-836 [PMID: 21222159 DOI: 10.1007/s10620-010-1544-3]

11 **Whitten KE**, Leach ST, Bohane TD, Woodhead HJ, Day AS. Effect of exclusive enteral nutrition on bone turnover in children with Crohn's disease. *J Gastroenterol* 2010; **45**: 399-405 [PMID: 19957194 DOI: 10.1007/s00535-009-0165-0]

12 **Beattie RM**, Schiffrin EJ, Donnet-Hughes A, Huggett AC, Domizio P, MacDonald TT, Walker-Smith JA. Polymeric nutrition as the primary therapy in children with small bowel Crohn's disease. *Aliment Pharmacol Ther* 1994; **8**: 609-615 [PMID: 7696450]

13 **Afzal NA**, Van Der Zaag-Loonen HJ, Arnaud-Battandier F, Davies S, Murch S, Derkx B, Heuschkel R, Fell JM. Improvement in quality of life of children with acute Crohn's disease does not parallel mucosal healing after treatment with exclusive enteral nutrition. *Aliment Pharmacol Ther* 2004; **20**: 167-172 [PMID: 15233696 DOI: 10.1111/j.1365-2036.2004.02002.x]

14 **Day AS**, Whitten KE, Sidler M, Lemberg DA. Systematic review: nutritional therapy in paediatric Crohn's disease. *Aliment Pharmacol Ther* 2008; **27**: 293-307 [PMID: 18045244 DOI: 10.1111/j.1365-2036.2007.03578.x]

15 **Takagi S**, Utsunomiya K, Kuriyama S, Yokoyama H, Takahashi S, Iwabuchi M, Takahashi H, Takahashi S, Kinouchi Y, Hiwatashi N, Funayama Y, Sasaki I, Tsuji I, Shimosegawa T. Effectiveness of an 'half elemental diet' as maintenance therapy for Crohn's disease: A randomized-controlled trial. *Aliment Pharmacol Ther* 2006; **24**: 1333-1340 [PMID: 17059514 DOI: 10.1111/j.1365-2036.2006.03120.x]

16 **Critch J**, Day AS, Otley A, King-Moore C, Teitelbaum JE, Shashidhar H. Use of enteral nutrition for the control of intestinal inflammation in pediatric Crohn disease. *J Pediatr Gastroenterol Nutr* 2012; **54**: 298-305 [PMID: 22002478 DOI: 10.1097/MPG.0b013e318235b397]

17 **Lichtenstein GR**, Hanauer SB, Sandborn WJ. Management of Crohn's disease in adults. *Am J Gastroenterol* 2009; **104**: 465-83; quiz 464, 484 [PMID: 19174807 DOI: 10.1038/ajg.2008.168]

18 **Zachos M**, Tondeur M, Griffiths AM. Enteral nutritional therapy for induction of remission in Crohn's disease. *Cochrane Database Syst Rev* 2007; (**1**): CD000542 [PMID: 17253452]

19 **Engelman JL**, Black L, Murphy GM, Sladen GE. Comparison of a semi elemental diet (Peptamen) with prednisolone in the primary-treatment of active ileal Crohn's disease. *Gastroenterology* 1993; **104**: A697-A697

20 **Mantzaris GJ**, Archavlis E, Amperiadis P, Kourtessas D, Triantafyllou G. A randomized prospective trial in active Crohn's disease comparing a polymeric diet, prednisolone, and a polymeric diet plus prednisolone. *Gastroenterology* 1996; **110**: A955-A955

21 **Gorard DA**, Hunt JB, Payne-James JJ, Palmer KR, Rees RG, Clark ML, Farthing MJ, Misiewicz JJ, Silk DB. Initial response and subsequent course of Crohn's disease treated with elemental diet or prednisolone. *Gut* 1993; **34**: 1198-1202 [PMID: 8406153 DOI: 10.1136/gut.34.9.1198]

22 **O'Moráin C**, Segal AW, Levi AJ. Elemental diet as primary treatment of acute Crohn's disease: a controlled trial. *Br Med J* (Clin Res Ed) 1984; **288**: 1859-1862 [PMID: 6428577]

23 **González-Huix F**, de León R, Fernández-Bañares F, Esteve M, Cabré E, Acero D, Abad-Lacruz A, Figa M, Guilera M, Planas R. Polymeric enteral diets as primary treatment of active Crohn's disease: a prospective steroid controlled trial. *Gut* 1993; **34**: 778-782 [PMID: 8314510 DOI: 10.1136/gut.34.6.778]

24 **Zoli G**, Carè M, Parazza M, Spanò C, Biagi PL, Bernardi M, Gasbarrini G. A randomized controlled study comparing elemental diet and steroid treatment in Crohn's disease. *Aliment Pharmacol Ther* 1997; **11**: 735-740 [PMID: 9305483 DOI: 10.1046/j.1365-2036.1997.t01-1-00192.x]

25 **Lindor KD**, Fleming CR, Burnes JU, Nelson JK, Ilstrup DM. A randomized prospective trial comparing a defined formula diet, corticosteroids, and a defined formula diet plus corticosteroids in active Crohn's disease. *Mayo Clin Proc* 1992; **67**: 328-333 [PMID: 1548947]

26 **Okada M**, Yao T, Yamamoto T, Takenaka K, Imamura K, Maeda K, Fujita K. Controlled trial comparing an elemental diet with prednisolone in the treatment of active Crohn's disease. *Hepatogastroenterology* 1990; **37**: 72-80 [PMID: 2179093]

27 **Gassull MA**, Fernández-Bañares F, Cabré E, Papo M, Giaffer MH, Sánchez-Lombraña JL, Richart C, Malchow H, González-Huix F, Esteve M. Fat composition may be a clue to explain the primary therapeutic effect of enteral nutrition in Crohn's disease: results of a double blind randomised multicentre European trial. *Gut* 2002; **51**: 164-168 [PMID: 12117873 DOI: 10.1136/gut.51.2.164]

28 **Lochs H**, Steinhardt HJ, Klaus-Wentz B, Zeitz M, Vogelsang H, Sommer H, Fleig WE, Bauer P, Schirrmeister J, Malchow H. Comparison of enteral nutrition and drug treatment in active Crohn's disease. Results of the European Cooperative Crohn's Disease Study. IV. *Gastroenterology* 1991; **101**: 881-888 [PMID: 1679736]

29 **Malchow H**, Steinhardt HJ, Lorenz-Meyer H, Strohm WD, Rasmussen S, Sommer H, Jarnum S, Brandes JW, Leonhardt H, Ewe K. Feasibility and effectiveness of a defined-formula diet regimen in treating active Crohn's disease. European Cooperative Crohn's Disease Study III. *Scand J Gastroenterol* 1990; **25**: 235-244 [PMID: 1969678]

30 **Vermeire S**, Schreiber S, Sandborn WJ, Dubois C, Rutgeerts P. Correlation between the Crohn's disease activity and Harvey-Bradshaw indices in assessing Crohn's disease severity. *Clin Gastroenterol Hepatol* 2010; **8**: 357-363 [PMID: 20096379 DOI: 10.1016/j.cgh.2010.01.001]

31 **van Hees PA**, van Elteren PH, van Lier HJ, van Tongeren JH. An index of inflammatory activity in patients with Crohn's disease. *Gut* 1980; **21**: 279-286 [PMID: 7429289 DOI: 10.1136/gut.21.4.279]

32 **Panaccione R**, Ghosh S. Optimal use of biologics in the management of Crohn's disease. *Therap Adv Gastroenterol* 2010; **3**: 179-189 [PMID: 21180600 DOI: 10.1177/1756283X09357579]

33 **Fascì Spurio F**, Aratari A, Margagnoni G, Doddato MT, Papi C. Early treatment in Crohn's disease: do we have enough evidence to reverse the therapeutic pyramid? *J Gastrointestin Liver Dis* 2012; **21**: 67-73 [PMID: 22457862]

34 **Grogan JL**, Casson DH, Terry A, Burdge GC, El-Matary W, Dalzell AM. Enteral feeding therapy for newly diagnosed pediatric Crohn's disease: a double-blind randomized controlled trial with two years follow-up. *Inflamm Bowel Dis* 2012; **18**: 246-253 [PMID: 21425210 DOI: 10.1002/ibd.21690]

35 **Tarrant KM**, Barclay ML, Frampton CM, Gearry RB. Perianal disease predicts changes in Crohn's disease phenotype-results of a population-based study of inflammatory bowel disease phenotype. *Am J Gastroenterol* 2008; **103**: 3082-3093 [PMID: 19086959 DOI: 10.1111/j.1572-0241.2008.02212.x]

36 **Baert F**, Moortgat L, Van Assche G, Caenepeel P, Vergauwe P, De Vos M, Stokkers P, Hommes D, Rutgeerts P, Vermeire S, D'Haens G. Mucosal healing predicts sustained clinical remission in patients with early-stage Crohn's disease. *Gastroenterology* 2010; **138**: 463-48; quiz 463-48 [PMID: 19818785 DOI: 10.1053/j.gastro.2009.09.056]

37 **Berni Canani R**, Terrin G, Borrelli O, Romano MT, Man-

guso F, Coruzzo A, D'Armiento F, Romeo EF, Cucchiara S. Short- and long-term therapeutic efficacy of nutritional therapy and corticosteroids in paediatric Crohn's disease. *Dig Liver Dis* 2006; **38**: 381-387 [PMID: 16301010 DOI: 10.1016/j.dld.2005.10.005]

38 **Rubio A**, Pigneur B, Garnier-Lengliné H, Talbotec C, Schmitz J, Canioni D, Goulet O, Ruemmele FM. The efficacy of exclusive nutritional therapy in paediatric Crohn's disease, comparing fractionated oral vs. continuous enteral feeding. *Aliment Pharmacol Ther* 2011; **33**: 1332-1339 [PMID: 21507029 DOI: 10.1111/j.1365-2036.2011.04662.x]

39 **Wong S**, Lemberg DA, Day AS. Exclusive enteral nutrition in the management of perianal Crohn's disease in children. *J Dig Dis* 2010; **11**: 185-188 [PMID: 20579222 DOI: 10.1111/j.1751-2980.2010.00434.x]

40 **Verma S**, Brown S, Kirkwood B, Giaffer MH. Polymeric versus elemental diet as primary treatment in active Crohn's disease: a randomized, double-blind trial. *Am J Gastroenterol* 2000; **95**: 735-739 [PMID: 10710067 DOI: 10.1016/s0002-9270(99)00586-9]

41 **Sakurai T**, Matsui T, Yao T, Takagi Y, Hirai F, Aoyagi K, Okada M. Short-term efficacy of enteral nutrition in the treatment of active Crohn's disease: a randomized, controlled trial comparing nutrient formulas. *JPEN J Parenter Enteral Nutr* 2002; **26**: 98-103 [PMID: 11871742 DOI: 10.1177/014860710202600298]

42 **Mukai J**, Miyanaga Y, Ishizaka T, Asaka K, Nakai Y, Tsuji E, Uchida T. Quantitative taste evaluation of total enteral nutrients. *Chem Pharm Bull* (Tokyo) 2004; **52**: 1416-1421 [PMID: 15577236 DOI: 10.1248/cpb.52.1416]

43 **De Araujo IE**, Rolls ET. Representation in the human brain of food texture and oral fat. *J Neurosci* 2004; **24**: 3086-3093 [PMID: 15044548 DOI: 10.1523/jneurosci.0130-04.2004]

44 **Rodrigues AF**, Johnson T, Davies P, Murphy MS. Does polymeric formula improve adherence to liquid diet therapy in children with active Crohn's disease? *Arch Dis Child* 2007; **92**: 767-770 [PMID: 17475695 DOI: 10.1136/adc.2006.103416]

P- Reviewers: Campo SMA, Swaminath A **S- Editor:** Wen LL
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.v19.i43.7661

World J Gastroenterol 2013 November 21; 19(43): 7661-7670
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

MINIREVIEWS

Diagnosis of IgG4-related sclerosing cholangitis

Takahiro Nakazawa, Itaru Naitoh, Kazuki Hayashi, Katsuyuki Miyabe, Shuya Simizu, Takashi Joh

Takahiro Nakazawa, Itaru Naitoh, Kazuki Hayashi, Katsuyuki Miyabe, Shuya Simizu, Takashi Joh, Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601, Japan
Author contributions: All authors contributed to this work.
Correspondence to: Takahiro Nakazawa, MD, Department of Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan. tnakazaw@med.nagoya-cu.ac.jp
Telephone: +81-52-8538211 Fax: +81-52-8520952
Received: June 20, 2013 Revised: September 17, 2013
Accepted: September 29, 2013
Published online: November 21, 2013

Abstract

IgG4-related sclerosing cholangitis (IgG4-SC) is often associated with autoimmune pancreatitis. However, the diffuse cholangiographic abnormalities observed in IgG4-SC may resemble those observed in primary sclerosing cholangitis (PSC), and the presence of segmental stenosis suggests cholangiocarcinoma (CC). IgG4-SC responds well to steroid therapy, whereas PSC is only effectively treated with liver transplantation and CC requires surgical intervention. Since IgG4-SC was first described, it has become a third distinct clinical entity of sclerosing cholangitis. The aim of this review was to introduce the diagnostic methods for IgG4-SC. IgG4-SC should be carefully diagnosed based on a combination of characteristic clinical, serological, morphological, and histopathological features after cholangiographic classification and targeting of a disease for differential diagnosis. When intrapancreatic stenosis is detected, pancreatic cancer or CC should be ruled out. If multiple intrahepatic stenoses are evident, PSC should be distinguished on the basis of cholangiographic findings and liver biopsy with IgG4 immunostaining. Associated inflammatory bowel disease is suggestive of PSC. If stenosis is demonstrated in the hepatic hilar region, CC should be discriminated by ultrasonography, intraductal ultrasonography, bile duct biopsy, and a higher cutoff serum IgG4 level of 182 mg/dL.

Key words: IgG4-related sclerosing cholangitis; Primary sclerosing cholangitis; IgG4; Sclerosing cholangitis

Core tip: IgG4-related sclerosing cholangitis (IgG4-SC) has become a third distinct clinical entity of sclerosing cholangitis. The diffuse cholangiographic abnormalities observed in IgG4-SC may resemble those observed in primary sclerosing cholangitis (PSC), and the presence of segmental stenosis suggests cholangiocarcinoma (CC). IgG4-SC responds well to steroid therapy, whereas PSC is only effectively treated with liver transplantation, and CC requires surgical intervention. IgG4-SC should be carefully diagnosed based on a combination of characteristic clinical, serological, morphological, and histopathological features after cholangiographic classification and targeting of a disease for differential diagnosis.

Nakazawa T, Naitoh I, Hayashi K, Miyabe K, Simizu S, Joh T. Diagnosis of IgG4-related sclerosing cholangitis. *World J Gastroenterol* 2013; 19(43): 7661-7670 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7661.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7661

INTRODUCTION

IgG4-related sclerosing cholangitis (IgG4-SC) is a characteristic type of sclerosing cholangitis, with an unknown pathogenic mechanism. Patients with IgG4-SC display increased serum IgG4 levels[1] and dense infiltration of IgG4-positive plasma cells with extensive fibrosis in the bile duct wall[2]. Circular and symmetrical thickening of the bile duct wall is observed in the areas without stenosis that appear normal on cholangiography, as well as in the stenotic areas[3]. IgG4-SC has been recently recognized as an IgG4-related disease. IgG4-SC is frequently associated with autoimmune pancreatitis (AIP). IgG4-related dacryoadenitis/sialadenitis and IgG4-related retroperitoneal

fibrosis are also occasionally observed in IgG4-SC[4-7]. However, some IgG4-SC cases do not involve other organs[8]. IgG4-SC is most common in elderly men. Obstructive jaundice is frequently observed in IgG4-SC. The clinical and radiological features of IgG4-SC are resolved by steroid therapy, although its long-term prognosis is not clear[9-12]. The diffuse cholangiographic abnormalities observed in IgG4-SC may resemble those observed in primary sclerosing cholangitis (PSC), and the presence of segmental stenosis suggests cholangiocarcinoma (CC). IgG4-SC responds well to steroid therapy, whereas PSC is effectively treated only with liver transplantation, and CC requires surgical intervention. It is also necessary to rule out secondary sclerosing cholangitis (SSC) caused by diseases with an obvious pathogenesis.

Precise diagnosis is needed before choosing appropriate treatments. Therefore, this paper provides a review of the clinical and pathological characteristics of IgG4-SC, focusing on its differential diagnosis from other biliary diseases such as PSC and CC.

CLASSIFICATION OF SC

SC has been classified into two categories: PSC and SSC. IgG4-SC has sometimes been described as an isolated biliary tract lesion, even in the absence of pancreatic involvement, and has thus been established as a distinct clinical entity. Therefore, we propose that SC should now be classified into three categories: PSC, IgG4-SC, and SSC. We have identified three reasons why IgG4-SC should be considered independent of other forms of SSC. First, steroid therapy is highly effective for IgG4-SC, which is in contrast to the other types of SC. Second, in comparison with the other forms, IgG4-SC is the most frequently encountered in daily clinical practice. Third, the characteristics of IgG4-SC need to be fully discriminated from those of the other three intractable diseases, that is, pancreatic cancer (PCa), PSC, and CC.

With regard to the diagnosis of SC, SSC should be initially ruled out. Thereafter, IgG4-SC should be suspected, the serum IgG4 level measured, and further exploration for pancreatic involvement or other IgG4-related systemic disease, conducted. Finally, compatibility with the PSC criteria should be ascertained.

PSC

The following diagnostic criteria for PSC, which were proposed by the Mayo Clinic, have been widely used[13]: typical cholangiographic abnormalities involving the intrabiliary and extrabiliary trees, compatible clinical and biochemical findings, and exclusion of other causes of SSC. Liver biopsy had been used in the past to help confirm diagnosis, but its diagnostic specificity and sensitivity have become controversial. Nevertheless, liver biopsy is useful in the diagnosis of small duct PSC, for patients with suspected PSC but normal cholangiographic findings, and for the exclusion of other cholestatic diseases.

Characteristic inflammatory bowel diseases (IBDs) are frequently observed in PSC patients. Standard ursodeoxycholic acid doses lead to improvements in biochemical abnormalities but not in histological findings, cholangiographic appearance, or patient survival. Liver transplantation is considered effective for end-stage liver disease because of PSC and is associated with improved patient survival. PSC usually leads to cirrhosis, with a mean survival time of 12-17 years.

IgG4-SC

Recently, IgG4-SC has attracted much attention with the emergence of clinical characteristics that distinguish it as a new clinical entity. The diffuse cholangiographic abnormalities observed in association with AIP may resemble those observed in PSC, and the segmental stenosis suggest CC. IgG4-SC responds well to steroid therapy compared with the other two types of SC.

We have previously reported on the differences between IgG4-SC and PSC[9]. The age at clinical onset is significantly older for patients with IgG4-SC. Among the chief complaints in IgG4-SC, obstructive jaundice, reflecting marked concentric stenosis of the large bile duct, is most frequently observed. However, in Japan, patients with PSC who present without symptoms after liver injury are identified by physical examination[14].

An elevated serum IgG4 level is a characteristic feature of IgG4-SC[15]. In patients with IgG4-SC, the pancreas is the most common organ involved other than the liver. Patients with IgG4-SC have multiorgan involvement, including sclerosing sialadenitis, retroperitoneal fibrosis, and mediastinal lymphadenopathy[4-7].

SSC

SSC is a chronic cholestatic biliary disease that can develop after a diverse range of insults to the biliary tree. SSC is considered to develop as a consequence of known injuries or secondary to pathological processes of the biliary tree. The etiology of SSC can usually be identified, although the exact pathogenesis often remains speculative. The most frequently described causes of SSC are long-standing biliary obstruction, surgical trauma to the bile duct, and ischemic injury to the biliary tree in liver allografts.

The different types of SSC have been described in the diagnostic criteria established by the Mayo Clinic[13]. Two reviews of SSC cases have been published[16,17]. IgG4-SC was previously classified into SSC. We classified the etiology of SSC based on three review articles[13,16,17] (Table 1). There are few studies comparing patients with SSC and PSC. A 10-year retrospective review (1992-2002) by the Mayo Clinic identified 31 patients with SSC[18]. The documented etiologies in their series included surgical trauma from cholecystectomy, intraductal stones, recurrent pancreatitis, and abdominal injury. Nine of their patients with SSC ultimately required liver transplantation, and four died. In their series, when compared with matched controls with PSC, the patient transplant-free survival was significantly shorter.

Table 1 Etiology of secondary sclerosing cholangitis

Congenital	Caroli's disease
	Cystic fibrosis
Chronic obstruction	Choledocholithiasis
	Biliary stricutures (secondary to surgical trauma, choronic pancreatitis)
	Anastomotic stricutures in liver graft
	Neoplasms (benign, malignant, metastatic)
Infectious	Bacterial cholangitis
	Recurrent pyogenic cholangitis
	Parasitic infection (cryptosporidiosis, microsporidiosis)
	Cytomegalovirus infection
Toxic	Accidental alcohol, formaldehyde, hypertonic saline instillation in the bile ducts
Immunologic	Eosinophilic cholangitis
	Acquired immunodeficiency
Ischemic	Vascular trauma
	Post-traumatic sclerosing cholangitis
	Post-transplantation hepatic artery thrombosis
	Hepatic allograft rejection (acute, chronic)
	Intra-arterial, chemotherapy-related injury
	Transcatheter arterial embolization therapy
Infiltrative disorders	Systemic vasculitis
	Amyloidosis
	Radiation injury
	Sarcoidosis
	Systemic mastocytosis
	Hypereosinophilic syndrome
	Hodgkin's disease

IgG4-SC DIAGNOSIS

Cholangiographic classification

IgG4-SC displays various cholangiographic features similar to those of PCa, PSC, and CC. The characteristic features of IgG4-SC can be classified into four types based on the stricture regions revealed by cholangiography and differential diagnosis (Figure 1)[19,20]. Type 1 IgG4-SC displays stenosis only in the lower part of the common bile duct and thus should be differentiated from chronic pancreatitis, PCa, and CC. Type 2 IgG4-SC, in which stenosis is diffusely distributed throughout the intrahepatic and extrahepatic bile ducts, should be differentiated from PSC and is further subdivided into two subtypes: type 2a, characterized with narrowing of the intrahepatic bile ducts with prestenotic dilation; and type 2b, characterized by the narrowing of the intrahepatic bile ducts without prestenotic dilation and reduced bile duct branches, which is caused by marked lymphocytic and plasmacyte infiltrations into the peripheral bile ducts. Type 3 IgG4-SC is characterized by stenosis in the hilar hepatic lesions and the lower part of the common bile duct. Type 4 IgG4-SC presents with strictures of the bile duct only in the hilar hepatic lesions. The cholangiographic findings of types 3 and 4 IgG4-SC should be discriminated from those of CC.

Serum IgG4 level

Serum IgG4 level has been reported to be a useful marker for discriminating AIP from other pancreatic diseases. A cutoff IgG4 level of 135 mg/dL is widely used as part of the diagnostic criteria for AIP. However, twice the upper limit of normal is also recommended to discriminate AIP from PCa. In the international consensus diagnostic criteria for AIP, once or twice the upper limit of normal is included in levels 1 and 2 diagnoses, respectively[21].

Only a few reports have been published concerning the cutoff IgG4 level in the diagnosis of IgG4-SC. We published for the first time, the diagnostic criteria for IgG4-SC based on a comparative study[22]. The cutoff IgG4 level of 135 mg/dL is useful in discriminating IgG4-SC from PCa and PSC. However, this cutoff level displayed lower specificity in discriminating IgG4-SC from CC. Oseini *et al*[23] evaluated the utility of serum IgG4 level in distinguishing IgG4-SC from CC. They reported that among their 126 patients with CC, 17 (13.5%) had elevated (> 140 mg/dL) and four (3.2%) had a > 2-fold increase (> 280 mg/dL) in serum IgG4 levels. PSC was present in 31/126 CC patients, of whom seven (22.6%) had an elevated serum IgG4 level. The authors concluded that some patients with CC, particularly PSC, had elevated serum IgG4 levels and diagnosis using a twofold higher cutoff serum IgG4 level may not reliably distinguish IgG4-SC from CC. However, a cutoff level fourfold higher than the upper limit of normal had 100% specificity for IgG4-SC.

We recently established a cutoff serum IgG4 level to differentiate IgG4-SC from the three other diseases (type 1 IgG4-SC *vs* PCa, type 2 IgG4-SC *vs* PSC, and type 3 IgG4-SC *vs* CC) using serum IgG4 levels measured in nine Japanese high-volume centers[24]. The cutoff obtained from receiver operator characteristic (ROC) curves displayed similar sensitivity and specificity to those of 135 mg/dL when all the IgG4-SC cases and controls were compared. However, a new cutoff value was established when IgG4-SC subgroups and controls were compared. A cutoff level of 182 mg/dL can increase the specificity to 96.6% (a 4.7% increase) for distinguishing types 3 and 4 IgG4-SC from CC. A cutoff level of 207 mg/dL might be useful for completely distinguishing types 3 and 4 IgG4-SC from CC.

Alswat *et al*[25] demonstrated that serum IgG4 levels could efficiently detect patients with IgG4-SC after excluding SC patients with AIP. However, previously reported IgG4-SC cases without pancreatic involvement displayed no marked increase in serum IgG4 level compared with patients with AIP-associated IgG4-SC. Hamano *et al*[8] demonstrated modestly high serum IgG4 levels (119, 122, and 195 mg/dL) in three IgG4-SC cases without an obvious pancreatic mass.

Elevated serum IgG4 level is considered a characteristic feature of IgG4-SC[1]. However, Mendes *et al*[26] measured the serum IgG4 level in 127 patients with PSC and found that it was elevated in 12 (9%). The patients with elevated IgG4 levels had higher levels of total bilirubin and alkaline phosphatase, higher PSC Mayo risk scores, and lower incidence of IBD. It is important to note that the time to liver transplantation was shorter in

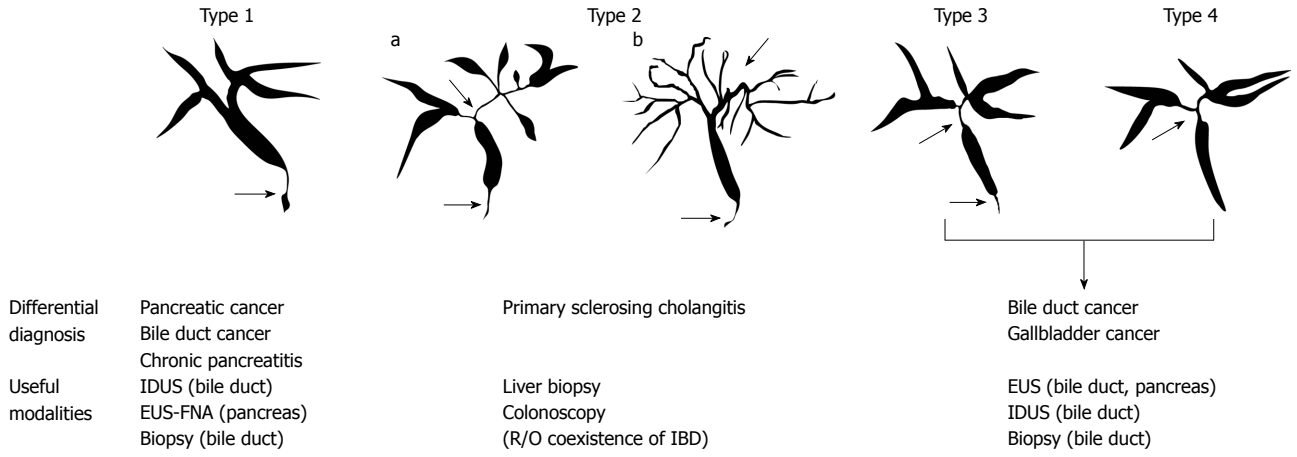


Figure 1 Cholangiographic classification of IgG4-related sclerosing cholangitis and differential diagnosis. Stenosis is located only in the lower part of the common bile duct in type 1; stenosis is diffusely distributed in the intra-and extra-hepatic bile ducts in type 2. Type 2 is further subdivided into two. Extended narrowing of the intrahepatic bile ducts with prestenotic dilation is widely distributed in type 2a. Narrowing of the intrahepatic bile ducts without prestenotic dilation and reduced bile duct branches are widely distributed in type 2b; stenosis is detected in both the hilar hepatic lesions and the lower part of the common bile ducts in type 3; strictures of the bile duct are detected only in the hilar hepatic lesions in type 4. IDUS: Intraductal ultrasonography; EUS-FNA: Endoscopic ultrasound-guided fine needle aspiration; IBD: Inflammatory bowel disease.

the patients with elevated IgG4 levels (1.7 years *vs* 6.5 years, $P = 0.0009$). As only one of the patients in their series had an abnormal pancreatogram, the documented cases appeared to conform to the diagnosis of IgG4-SC. Therefore, clinical trials in which patients with PSC are evaluated for IgG4 and patients presenting elevated levels are treated with corticosteroids should be considered.

Other organ involvement

The concept of IgG4-related disease has been established internationally[27]. IgG4-SC is included in the IgG4-related disease category. Serum IgG4 level elevation and tissue infiltration with IgG4-positive plasma cells are common threads that connect a variety of apparently disparate conditions observed previously in multiple organs. Certain clinical and pathological features help define IgG4-related disease and distinguish it from its potential mimics. IgG4-related disease is a fibroinflammatory condition characterized by a tendency to form tumefactive lesions, a dense lymphoplasmacytic infiltrate rich in IgG4-positive plasma cells, storiform fibrosis, frequent but not invariable elevations in serum IgG4 level, and a swift initial response to glucocorticoids, provided that tissue fibrosis has not supervened.

The pancreas was the first organ in which IgG4-related disease was identified, but the disease has now been described in virtually every organ system: the biliary tree, salivary glands, orbital tissues (*e.g.*, lacrimal gland, extraocular muscles, and retrobulbar space), kidneys, lungs, lymph nodes, meninges, aorta, breast, prostate, thyroid gland, pericardium, retroperitoneum, and skin.

Association with AIP is a useful finding in the diagnosis of IgG4-SC. In one study, 59 (95%) of 62 patients with IgG4-SC had associated AIP, with high prevalence[21]. Ghazale *et al*[15] reported an incidence rate of AIP association of 92% in 53 patients with IgG4-SC, which was a comparatively large sample. However, focal-type AIP sometimes displays similar imaging findings to those of PCa, making discrimination between the two diseases difficult[28]. The sensitivity rates of diagnostic criteria for AIP have been reported to range from 80% to 92%[29]. Therefore, useful diagnostic criteria need to be established for IgG4-SC when it is not associated with AIP or when diagnosis of AIP is unclear. IgG4-SC is occasionally associated with other systemic IgG4-related diseases including symmetrical dacryoadenitis/sialadenitis and retroperitoneal fibrosis. These associations are helpful in the accurate diagnosis of IgG4-SC.

Pathological features

In IgG4-SC, fibroinflammatory involvement is observed mainly in the submucosa of the bile duct wall, whereas the epithelium of the bile duct is intact[2]. However, slight injury and/or neutrophil infiltration are occasionally observed in IgG4-SC with associated secondary cholangitis. PSC should be ruled out if inflammation is observed, particularly in the epithelium of the bile duct wall. The characteristic pathological findings of IgG4-SC were first reported as "lymphoplasmacytic sclerosing pancreatitis with cholangitis"[30]. Abraham *et al*[31] reported frequent fibroinflammatory involvement of the gallbladder and common bile duct in patients with lymphoplasmacytic sclerosing pancreatitis. Zen *et al*[2] revealed that the bile duct wall in IgG4-SC had pathological features similar to those of AIP, displaying dense infiltrations of lymphocytes and IgG4-positive plasma cells, with extensive fibrosis and obliterative phlebitis. They classified IgG4-SC into six categories according to the extent of inflammation and association with an inflammatory pseudotumor. IgG4-positive plasma cells are sparse in the affected bile ducts in PSC, and the luminal side of the bile ducts, including lining biliary epithelial cells, is preferentially affected compared with IgG4-SC. In PSC, the fibrosis is denser and older, whereas in IgG4-SC, the entire bile

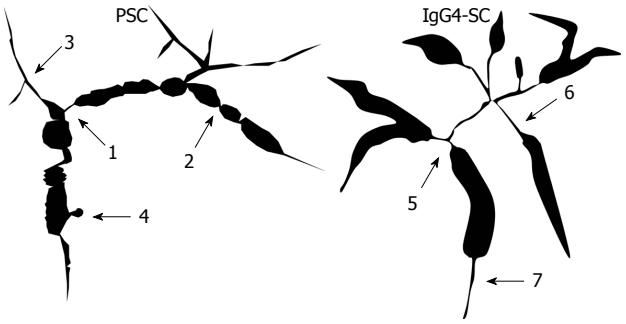


Figure 2 Schematic illustration of comparison of cholangiographic (primary sclerosing cholangitis *vs* IgG4-related sclerosing cholangitis) findings[28]. The schematic comparison of cholangiographic findings between IgG4-related sclerosing cholangitis (SC) and primary sclerosing cholangitis (PSC). IgG4-related SC displays segmental and long strictures and stricture of the lower common bile duct, whereas PSC displays band-like strictures (1-2 mm), beaded appearance (short and annular stricture alternating with normal or minimally dilated segments), pruned-tree appearance (diminished arbolization of intrahepatic duct and pruning), and diverticulum-like outpouching (outpouchings resembling diverticula, often protruding between adjacent strictures). 1: Band-like stricture; 2: Beaded appearance; 3: Pruned-tree appearance; 4: Dverticulum-like outpouching; 5: Segmental stricture; 6: Long stricture with prestenotic dilation; 7: Stricture of lower common bile duct.

duct wall and periductal tissue are affected. However, a recent study by Zhang *et al*[32] revealed that 23 (23%) of 98 liver explants with PSC had periductal infiltration with abundant IgG4-positive plasma cells [10/high-power field (HPF)] in the hilar area.

Differential diagnosis of IgG4-SC based on cholangiographic classification

IgG4-SC displays various cholangiographic features similar to those of PCa, PSC, and CC[9]. The differential diagnosis based on cholangiographic classification is sufficient in clinical practice because the useful modalities depend on the cholangiographic types (Figure 1)[20].

Type 1 IgG4-SC should be differentiated from chronic pancreatitis, PCa, and CC. The modalities useful for differential diagnosis are intraductal ultrasonography (IDUS)[3], endoscopic ultrasound-guided fine-needle aspiration for the pancreas[33], and cytological examination and/or biopsy of the bile duct[3,34].

Type 2 IgG4-SC should be differentiated from PSC. The modalities useful for differential diagnosis are cholangiography[35], evaluations for associated IBD[9,12], and liver biopsy[36,37]. Our discriminant analysis formula for cholangiographic features, including age, was able to discriminate type 2 IgG4-SC from PSC[35]. Band-like strictures, a beaded or "pruned tree" appearance, and diverticulum-like outpouching are significantly more frequent in PSC cases. In contrast, segmental strictures, long strictures with prestenotic dilation, and strictures of the lower common bile duct are significantly more common in IgG4-SC. These differences are illustrated in Figure 2. The characteristic cholangiographic features reflect the underlying pathological processes involved in each condition. In PSC, obliterative fibrosis is the main cause of biliary stenosis, creating short strictures. In contrast, in IgG4-SC, severe lymphoplasmacyte infiltration into bile ducts in the long region is the main cause of biliary stenosis, resulting in long strictures (Figure 3).

In contrast, Kalaitzakis *et al*[38] reported that diagnosing IgG4-SC by cholangiography displayed high specificity but poor sensitivity and may have led to the misdiagnosis of IgG4-SC as PSC or CC.

Associated ulcerative colitis is suggestive of PSC. IBD is present in only 0%-6% of patients with IgG4-SC[9,12,15]. IBD is usually not a feature associated with type 1 AIP, unlike the frequent association of IBD with type 2 AIP[23]. IBD associated with PSC represents a third phenotype in western countries[39]. Backwash ileitis, rectal sparing, and low disease activity appear to be features that characterize IBD when it is associated with PSC[39,40].

The histological features of IgG4-SC on liver biopsy are distinctive and, in conjunction with IgG4 immunohistochemical staining, help distinguish IgG4-SC from PSC[36,41]. We have already reported that liver needle biopsy is especially useful for distinguishing IgG4-SC from PSC in patients with cholangiographically evident intrahepatic biliary strictures[37]. Four (57%) of seven patients with type 2 IgG4-SC presented infiltration with $\geqslant$ 10 IgG4-positive plasma cells per HPF in liver biopsy samples, whereas none of the PSC patients presented this feature.

Types 3 and 4 IgG4-SC need to be discriminated from CC. The modalities useful for the differential diagnosis of types 3 and 4 IgG4-SC are endoscopic procedures[42] such as endoscopic ultrasonography, IDUS[3,43], cytological examination, and/or biopsy of the bile duct[3,34]. Although we described how type 2 IgG4-SC could be discriminated from PSC on the basis of characteristic cholangiographic features, cholangiography cannot discriminate the segmental stricture of types 3 and 4 IgG4-SC from CC. Therefore, we applied our discriminant analysis formula for cholangiographic features to discriminate between only type 2 IgG4-SC and PSC.

IDUS findings such as circular-symmetrical wall thickening, smooth outer margin, smooth inner margin, and homogeneous internal echo at the stenotic area were useful for the diagnosis of IgG4-SC. The most characteristic IDUS finding in the IgG4-SC cases was thickening of the bile duct wall, which appeared normal on cholangiography[3]. Bile duct wall thickening spread continuously from the intrapancreatic bile duct to the upper bile duct in most cases. To differentiate IgG4-SC from CC, 0.8-mm thickness of the bile duct wall that appeared normal on a cholangiogram was the best cutoff as indicated by ROC curves. The sensitivity, specificity, and accuracy were 95%, 90.9%, and 93.5%, respectively, when the cutoff value was 0.8 mm. No CC cases had a bile duct wall thicker than 1 mm. The sensitivity, specificity, and accuracy were 85%, 100%, and 87%, respectively, when the cutoff value was set at 1 mm. We considered a 1-mm thickness as an optimal cutoff wall thickness to completely exclude CC.

Ghazale *et al*[15] reported the usefulness of endoscopic biliary biopsy for diagnosis of IgG4-SC. They

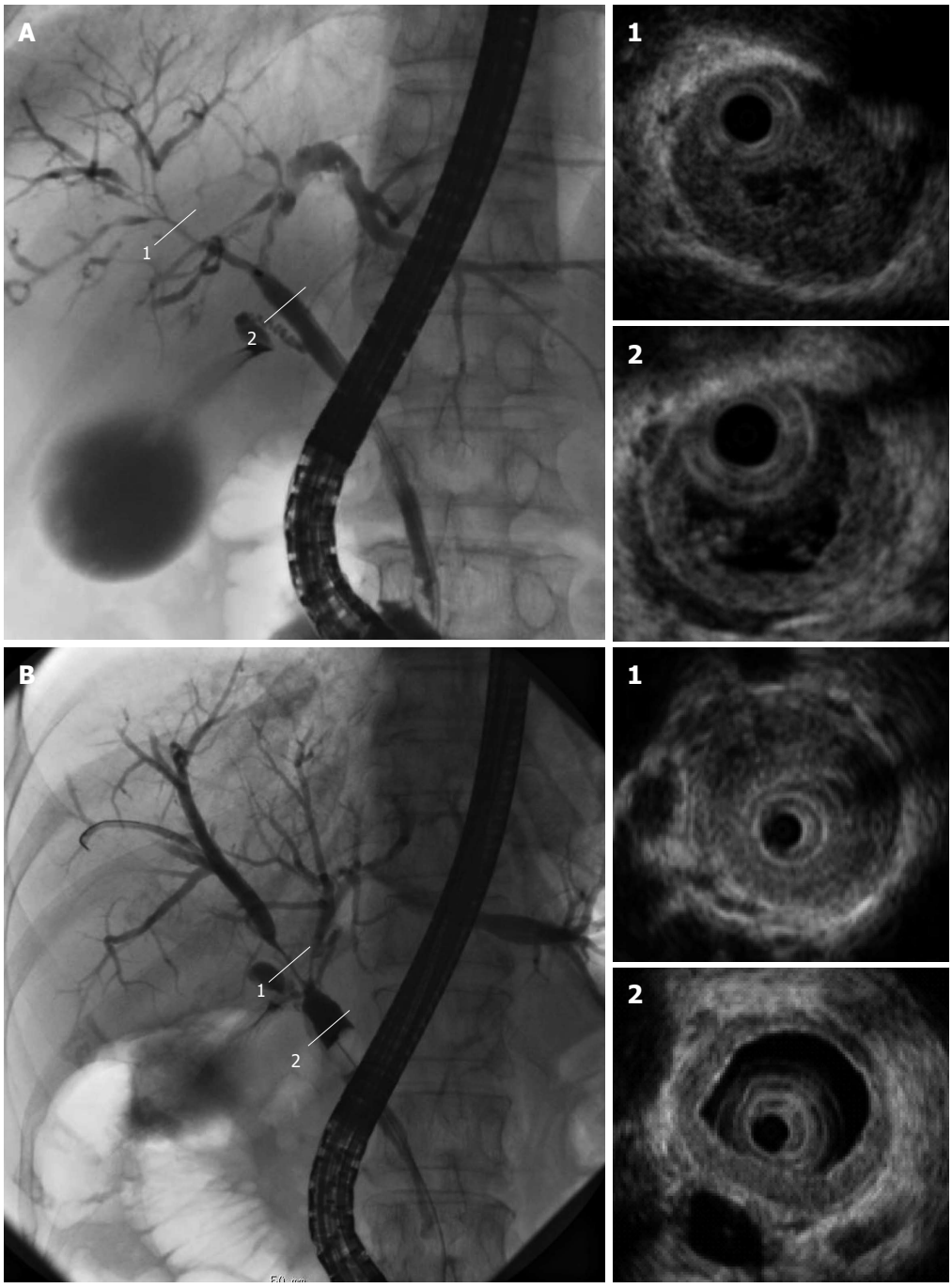


Figure 3 Cholangiogram displaying stenosis in the intrahepatic ducts (A-1) and hilar hepatic lesions (B-1); intraductal ultrasonography revealing bile duct wall thickening in areas with stenosis (1) and without (2).

reported that 14 (88%) of 16 patients had immunostaining results indicating abundant IgG4-positive cells (> 10 IgG4-positive cells/HPF) in bile duct biopsy specimens. Furthermore, they considered that the absence of malignant cells in the presence of an inflamed mucosa with many IgG4-positive plasma cells provided histological evidence to support the diagnosis of IgG4-SC. However, we were unable to diagnose any case as IgG4-SC on the basis of hematoxylin-eosin and elastin-van Gieson staining alone[3]. Abundant IgG4-positive plasma cells were observed in only three (18%) of 17 patients. We were able to diagnose IgG4-SC in only three patients (18%) on the basis of its characteristic histopathological features. However, it was possible to rule out CC by transpapillary biopsy. In addition, one of 11 CC cases presented with abundant IgG4-positive plasma cells. Zhang *et al*[32] also reported that abundant IgG4-positive plasma cells were evident in seven (18%) of 38 cases of CC. Harada *et al*[44] reported that CC cells could play the role of nonprofessional antigen-presenting cells and Foxp3-positive regulatory cells, inducing IgG4 reactions *via* the production of interleukin-10 indirectly and directly, respectively.

We could rule out CC by transpapillary biopsy. The superficial nature of endoscopic biopsy specimens lim-

its their usefulness for demonstrating the characteristic histological features of IgG4-SC. However, Kawakami *et al*[34] reported that the diagnostic rate from ampullary and bile duct biopsies was 52% (15/29 cases) based on the threshold of 10 IgG4-positive plasma cells per HPF, and that bile duct biopsy was valuable for patients with swelling of the pancreatic head. Ampullary biopsy is sometimes useful in the diagnosis of AIP and IgG4-SC[45,46].

Itoi *et al*[47] reported that cholangioscopy was useful in differentiating IgG4-SC from PSC and that monitoring patterns of proliferative vessels on video peroral cholangioscopy may be useful in differentiating IgG4-SC from CC.

Treatment and prognosis

Although some patients responded to biliary drainage or surgical resection, IgG4-SC displays a good response to steroid therapy, as is the case for pancreatic lesions.

Nishino *et al*[11] reported that bile duct stricture improved to various degrees in all 10 patients treated by steroid therapy but persisted in the lower part of the bile duct in four patients. Hirano *et al*[48] reported that steroid therapy could reduce AIP-related unfavorable events and that multivariate analysis indicated that steroid therapy and obstructive jaundice at onset were significant factors predictive of unfavorable events. Thus, early introduction of steroid therapy is recommended, especially for patients with obstructive jaundice. Ghazale *et al*[15] reported the clinical courses after steroid treatment (n = 30), surgical resection (n = 18), and conservative management (n = 5). Relapses occurred in 53% of cases after steroid withdrawal, whereas 44% relapsed after surgery and were further treated with steroids. The presence of proximal extrahepatic/intrahepatic strictures was predictive of relapse. Steroid therapy normalized liver enzyme levels in 61% of patients, and it was possible to remove biliary stents in 17 of 18 patients. Fifteen patients treated with steroids for relapse after steroid withdrawal responded to the treatment, and seven treated with additional immunomodulatory drugs reportedly remained in steroid-free remission. Topazian *et al*[49] reported that biliary strictures in one patient improved after rituximab therapy and thus the biliary stents were removed. However, the role of immunomodulatory drugs for relapse warrants further study. In one of our series, six of seven cases of IgG4-SC without steroid therapy and IgG levels > 2000 mg/dL were associated with significantly higher incidence of recurrence or progression[50].

Morphological and functional changes in the liver and bile ducts in IgG4-SC during long-term observation have not yet been reported. Our long-term follow-up of IgG4-SC cases without steroid therapy revealed that two patients developed portal obstruction and liver atrophy but no sign of liver cirrhosis or failure[51]. Ghazale *et al*[15] reported that four of 53 patients displayed portal hypertension and liver cirrhosis during their clinical courses. It is possible that persistent jaundice without steroid administration could result in liver failure, thus necessitating orthotopic liver transplantation. However, further study is needed to elucidate the long-term outcome of IgG4-SC.

Steroid trial

Although, generally, diagnosis should be made before starting therapy, a steroid trial is needed in some cases[52]. If a diagnosis cannot be clearly established in type 2 IgG4-SC, then a steroid trial is recommended. If malignancy is not confirmed by bile duct biopsy in types 3 and 4 IgG4-SC and bile duct wall thickening that appears normal on a cholangiogram, a steroid trial is an option. No reports on any steroid trial for IgG4-SC have been published thus far. A short-term steroid trial should be performed carefully only by specialists in pancreatic and biliary diseases. In addition, steroid pulse therapy is also an option[53]. Advanced-stage IgG4-SC may sometimes be unresponsive to steroid therapy because cases of AIP and IgG4-SC show a predominantly inflammatory nature at the early stage, followed by relatively less inflammation but marked fibrous scarring later. This should be kept in mind when attempting a steroid trial for IgG4-SC diagnosis[54]. The time frame for a steroid trial for IgG4-SC remains unknown. When a cholangiogram is indicative of type 1, 3 or 4 IgG4-SC, IgG4-SC should be discriminated from PCa or CC. It is important not to delay the timing of surgery by performing a long-term steroid trial. If a cholangiogram is indicative of type 2 IgG4-SC, IgG4-SC should be discriminated from PSC. Sufficient time should be devoted to a steroid trial only if an increased risk of biliary infection can be avoided.

Diagnostic criteria

Only three sets of diagnostic criteria for IgG4-SC have been proposed[15,20,24]. AIP should be clinically discriminated only from PCa. However, IgG4-SC should be discriminated from all of the three intractable diseases, that is, PCa, PSC, and CC. Therefore, diagnostic criteria that take into account the differential diagnosis between these three intractable diseases need to be established[22]. Our diagnostic criteria provide a concrete diagnostic algorithm for IgG4-SC (Figure 4). Association with AIP and other organ involvements are common useful diagnostic parameters in all three IgG4-SC types. Characteristic cholangiogram, liver biopsy and exclusion of IBD are useful parameters in type 2 IgG4-SC. IDUS findings, exclusion of malignancy by bile duct biopsy and a serum IgG4 cut-off level of 207 mg/dL were useful parameters in type 3 and 4 IgG4-SC. Although, generally, diagnosis should be made before starting therapy, a steroid trial is needed in some cases.

The HISORt criteria for the diagnosis of IgG4-SC[15] are based on the characteristic features of IgG4-SC on histological, imaging, and serological examination; other organ involvement; and response to steroid therapy, which parallel the HISORt criteria established for AIP[55].

The Research Committee of IgG4-related Diseases and the Research Committee of Intractable Diseases of Liver and Biliary Tract, in association with the Ministry

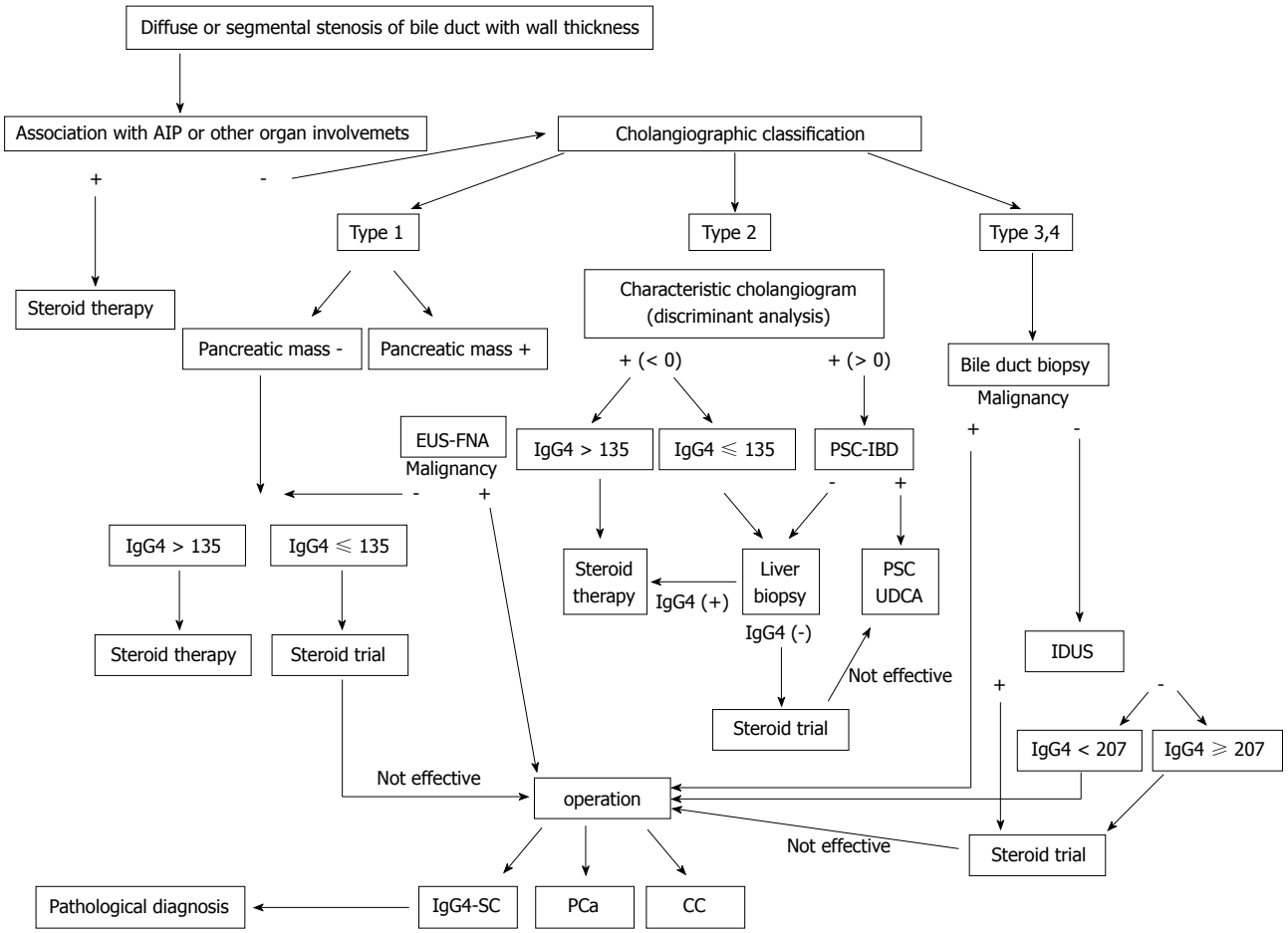


Figure 4 Algorithm for management of IgG4-related sclerosing cholangitis (cited from [22]). CC: Cholangiocarcinoma; PSC: Primary sclerosing cholangitis; IgG4-SC: IgG4-related sclerosing cholangitis; IDUS: Intraductal ultrasonography; EUS-FNA: Endoscopic ultrasound-guided fine needle aspiration; IBD: Inflammatory bowel disease; UDCA: Ursodeoxycholic acid.

of Health, Labor and Welfare, Japan, and the Japan Biliary Association, proposed clinical diagnostic criteria for IgG4-SC in 2012[20]. These diagnostic criteria also include the concept of differential diagnosis based on cholangiographic classification.

CONCLUSION

Since IgG4-SC was first described, it has become a third distinct clinical entity of SC. IgG4-SC should be carefully diagnosed based on a combination of characteristic clinical, serological, morphological, and histopathological features after cholangiographic classification and targeting of a disease for differential diagnosis.

REFERENCES

1 **Hamano H**, Kawa S, Horiuchi A, Unno H, Furuya N, Akamatsu T, Fukushima M, Nikaido T, Nakayama K, Usuda N, Kiyosawa K. High serum IgG4 concentrations in patients with sclerosing pancreatitis. *N Engl J Med* 2001; **344**: 732-738 [PMID: 11236777 DOI: 10.1056/NEJM200103083441001]

2 **Zen Y**, Harada K, Sasaki M, Sato Y, Tsuneyama K, Haratake J, Kurumaya H, Katayanagi K, Masuda S, Niwa H, Morimoto H, Miwa A, Uchiyama A, Portmann BC, Nakanuma Y. IgG4-related sclerosing cholangitis with and without hepatic inflammatory pseudotumor, and sclerosing pancreatitis-associated sclerosing cholangitis: do they belong to a spectrum of sclerosing pancreatitis? *Am J Surg Pathol* 2004; **28**: 1193-1203 [PMID: 15316319 DOI: 10.1097/01.pas.0000136449.37936.6c]

3 **Naitoh I**, Nakazawa T, Ohara H, Ando T, Hayashi K, Tanaka H, Okumura F, Takahashi S, Joh T. Endoscopic transpapillary intraductal ultrasonography and biopsy in the diagnosis of IgG4-related sclerosing cholangitis. *J Gastroenterol* 2009; **44**: 1147-1155 [PMID: 19636664 DOI: 10.1007/s00535-009-0108-9]

4 **Kamisawa T**, Funata N, Hayashi Y, Eishi Y, Koike M, Tsuruta K, Okamoto A, Egawa N, Nakajima H. A new clinicopathological entity of IgG4-related autoimmune disease. *J Gastroenterol* 2003; **38**: 982-984 [PMID: 14614606 DOI: 10.1007/s00535-003-1175-y]

5 **Ohara H**, Nakazawa T, Sano H, Ando T, Okamoto T, Takada H, Hayashi K, Kitajima Y, Nakao H, Joh T. Systemic extrapancreatic lesions associated with autoimmune pancreatitis. *Pancreas* 2005; **31**: 232-237 [PMID: 16163054 DOI: 10.1097/01.mpa.0000175178.85786.1d]

6 **Hamano H**, Arakura N, Muraki T, Ozaki Y, Kiyosawa K, Kawa S. Prevalence and distribution of extrapancreatic lesions complicating autoimmune pancreatitis. *J Gastroenterol* 2006; **41**: 1197-1205 [PMID: 17287899 DOI: 10.1007/s00535-006-1908-9]

7 **Naitoh I**, Nakazawa T, Ohara H, Ando T, Hayashi K, Tanaka H, Okumura F, Miyabe K, Yoshida M, Sano H, Takada H, Joh T. Clinical significance of extrapancreatic lesions in

autoimmune pancreatitis. *Pancreas* 2010; **39**: e1-e5 [PMID: 19924018 DOI: 10.1097/MPA.0b013e3181bd64a1]
8 **Hamano H**, Kawa S, Uehara T, Ochi Y, Takayama M, Komatsu K, Muraki T, Umino J, Kiyosawa K, Miyagawa S. Immunoglobulin G4-related lymphoplasmacytic sclerosing cholangitis that mimics infiltrating hilar cholangiocarcinoma: part of a spectrum of autoimmune pancreatitis? *Gastrointest Endosc* 2005; **62**: 152-157 [PMID: 15990840 DOI: 10.1016/S0016-5107(05)00561-4]
9 **Nakazawa T**, Ohara H, Sano H, Ando T, Aoki S, Kobayashi S, Okamoto T, Nomura T, Joh T, Itoh M. Clinical differences between primary sclerosing cholangitis and sclerosing cholangitis with autoimmune pancreatitis. *Pancreas* 2005; **30**: 20-25 [PMID: 15632695]
10 **Erkelens GW**, Vleggaar FP, Lesterhuis W, van Buuren HR, van der Werf SD. Sclerosing pancreato-cholangitis responsive to steroid therapy. *Lancet* 1999; **354**: 43-44 [PMID: 10406367 DOI: 10.1016/S0140-6736(99)00603-0]
11 **Nishino T**, Toki F, Oyama H, Oi I, Kobayashi M, Takasaki K, Shiratori K. Biliary tract involvement in autoimmune pancreatitis. *Pancreas* 2005; **30**: 76-82 [PMID: 15632703]
12 **Nishino T**, Oyama H, Hashimoto E, Toki F, Oi I, Kobayashi M, Shiratori K. Clinicopathological differentiation between sclerosing cholangitis with autoimmune pancreatitis and primary sclerosing cholangitis. *J Gastroenterol* 2007; **42**: 550-559 [PMID: 17653651]
13 **Lindor KD**, LaRusso NA. Primary sclerosing cholangitis. 10th ed. Schiff E, editor. Philadelphia: Lippincott Wiliams and Wilikins, 2007: 673-684
14 **Takikawa H**, Takamori Y, Tanaka A, Kurihara H, Nakanuma Y. Analysis of 388 cases of primary sclerosing cholangitis in Japan; Presence of a subgroup without pancreatic involvement in older patients. *Hepatol Res* 2004; **29**: 153-159 [PMID: 15203079 DOI: 10.1016/j.hepres.2004.03.006]
15 **Ghazale A**, Chari ST, Zhang L, Smyrk TC, Takahashi N, Levy MJ, Topazian MD, Clain JE, Pearson RK, Petersen BT, Vege SS, Lindor K, Farnell MB. Immunoglobulin G4-associated cholangitis: clinical profile and response to therapy. *Gastroenterology* 2008; **134**: 706-715 [PMID: 18222442 DOI: 10.1053/j.gastro.2007.12.009]
16 **Abdalian R**, Heathcote EJ. Sclerosing cholangitis: a focus on secondary causes. *Hepatology* 2006; **44**: 1063-1074 [PMID: 17058222 DOI: 10.1002/hep.21405]
17 **Ruemmele P**, Hofstaedter F, Gelbmann CM. Secondary sclerosing cholangitis. *Nat Rev Gastroenterol Hepatol* 2009; **6**: 287-295 [PMID: 19404269]
18 **Gossard AA**, Angulo P, Lindor KD. Secondary sclerosing cholangitis: a comparison to primary sclerosing cholangitis. *Am J Gastroenterol* 2005; **100**: 1330-1333 [PMID: 15929765 DOI: 10.1111/j.1572-0241.2005.41526.x]
19 **Nakazawa T**, Ohara H, Sano H, Ando T, Joh T. Schematic classification of sclerosing cholangitis with autoimmune pancreatitis by cholangiography. *Pancreas* 2006; **32**: 229 [PMID: 16552350 DOI: 10.1097/01.mpa.0000202941.85955.07]
20 **Ohara H**, Okazaki K, Tsubouchi H, Inui K, Kawa S, Kamisawa T, Tazuma S, Uchida K, Hirano K, Yoshida H, Nishino T, Ko SB, Mizuno N, Hamano H, Kanno A, Notohara K, Hasebe O, Nakazawa T, Nakanuma Y, Takikawa H. Clinical diagnostic criteria of IgG4-related sclerosing cholangitis 2012. *J Hepatobiliary Pancreat Sci* 2012; **19**: 536-542 [PMID: 22717980 DOI: 10.1007/s00534-012-0521-y]
21 **Shimosegawa T**, Chari ST, Frulloni L, Kamisawa T, Kawa S, Mino-Kenudson M, Kim MH, Klöppel G, Lerch MM, Löhr M, Notohara K, Okazaki K, Schneider A, Zhang L. International consensus diagnostic criteria for autoimmune pancreatitis: guidelines of the International Association of Pancreatology. *Pancreas* 2011; **40**: 352-358 [PMID: 21412117 DOI: 10.1097/MPA.0b013e3182142fd2]
22 **Nakazawa T**, Naitoh I, Hayashi K, Okumura F, Miyabe K, Yoshida M, Yamashita H, Ohara H, Joh T. Diagnostic criteria for IgG4-related sclerosing cholangitis based on cholangiographic classification. *J Gastroenterol* 2012; **47**: 79-87 [PMID: 21947649 DOI: 10.1007/s00535-011-0465-z]
23 **Oseini AM**, Chaiteerakij R, Shire AM, Ghazale A, Kaiya J, Moser CD, Aderca I, Mettler TA, Therneau TM, Zhang L, Takahashi N, Chari ST, Roberts LR. Utility of serum immunoglobulin G4 in distinguishing immunoglobulin G4-associated cholangitis from cholangiocarcinoma. *Hepatology* 2011; **54**: 940-948 [PMID: 21674559]
24 **Ohara H**, Nakazawa T, Kawa S, Kamisawa T, Shimosegawa T, Uchida K, Hirano K, Nishino T, Hamano H, Kanno A, Notohara K, Hasebe O, Muraki T, Ishida E, Naitoh I, Okazaki K. Establishment of a serum IgG4 cut-off value for the differential diagnosis of IgG4-related sclerosing cholangitis: a Japanese cohort. *J Gastroenterol Hepatol* 2013; **28**: 1247-1251 [PMID: 23621484 DOI: 10.1111/jgh.12248]
25 **Alswat K**, Al-Harthy N, Mazrani W, Alshumrani G, Jhaveri K, Hirschfield GM. The spectrum of sclerosing cholangitis and the relevance of IgG4 elevations in routine practice. *Am J Gastroenterol* 2012; **107**: 56-63 [PMID: 22068666 DOI: 10.1038/ajg.2011.375]
26 **Mendes FD**, Jorgensen R, Keach J, Katzmann JA, Smyrk T, Donlinger J, Chari S, Lindor KD. Elevated serum IgG4 concentration in patients with primary sclerosing cholangitis. *Am J Gastroenterol* 2006; **101**: 2070-2075 [PMID: 16879434 DOI: 10.1111/j.1572-0241.2006.00772.x]
27 **Stone JH**, Khosroshahi A, Deshpande V, Chan JK, Heathcote JG, Aalberse R, Azumi A, Bloch DB, Brugge WR, Carruthers MN, Cheuk W, Cornell L, Castillo CF, Ferry JA, Forcione D, Klöppel G, Hamilos DL, Kamisawa T, Kasashima S, Kawa S, Kawano M, Masaki Y, Notohara K, Okazaki K, Ryu JK, Saeki T, Sahani D, Sato Y, Smyrk T, Stone JR, Takahira M, Umehara H, Webster G, Yamamoto M, Yi E, Yoshino T, Zamboni G, Zen Y, Chari S. Recommendations for the nomenclature of IgG4-related disease and its individual organ system manifestations. *Arthritis Rheum* 2012; **64**: 3061-3067 [PMID: 22736240 DOI: 10.1002/art.34593]
28 **Nakazawa T**, Ohara H, Sano H, Ando T, Imai H, Takada H, Hayashi K, Kitajima Y, Joh T. Difficulty in diagnosing autoimmune pancreatitis by imaging findings. *Gastrointest Endosc* 2007; **65**: 99-108 [PMID: 17185087 DOI: 10.1016/j.gie.2006.03.929]
29 **Okazaki K**, Kawa S, Kamisawa T, Naruse S, Tanaka S, Nishimori I, Ohara H, Ito T, Kiriyama S, Inui K, Shimosegawa T, Koizumi M, Suda K, Shiratori K, Yamaguchi K, Yamaguchi T, Sugiyama M, Otsuki M. Clinical diagnostic criteria of autoimmune pancreatitis: revised proposal. *J Gastroenterol* 2006; **41**: 626-631 [PMID: 16932998 DOI: 10.1007/s00535-006-1868-0]
30 **Kawaguchi K**, Koike M, Tsuruta K, Okamoto A, Tabata I, Fujita N. Lymphoplasmacytic sclerosing pancreatitis with cholangitis: a variant of primary sclerosing cholangitis extensively involving pancreas. *Hum Pathol* 1991; **22**: 387-395 [PMID: 2050373 DOI: 10.1016/0046-8177(91)90087-6]
31 **Abraham SC**, Cruz-Correa M, Argani P, Furth EE, Hruban RH, Boitnott JK. Lymphoplasmacytic chronic cholecystitis and biliary tract disease in patients with lymphoplasmacytic sclerosing pancreatitis. *Am J Surg Pathol* 2003; **27**: 441-451 [PMID: 12657928 DOI: 10.1097/00000478-200304000-00003]
32 **Zhang L**, Lewis JT, Abraham SC, Smyrk TC, Leung S, Chari ST, Poterucha JJ, Rosen CB, Lohse CM, Katzmann JA, Wu TT. IgG4+ plasma cell infiltrates in liver explants with primary sclerosing cholangitis. *Am J Surg Pathol* 2010; **34**: 88-94 [PMID: 20035148 DOI: 10.1097/PAS.0b013e3181c6c09a]
33 **Mizuno N**, Bhatia V, Hosoda W, Sawaki A, Hoki N, Hara K, Takagi T, Ko SB, Yatabe Y, Goto H, Yamao K. Histological diagnosis of autoimmune pancreatitis using EUS-guided trucut biopsy: a comparison study with EUS-FNA. *J Gastroenterol* 2009; **44**: 742-750 [PMID: 19434362 DOI: 10.1007/s00535-009-0062-6]

34 **Kawakami H**, Zen Y, Kuwatani M, Eto K, Haba S, Yamato H, Shinada K, Kubota K, Asaka M. IgG4-related sclerosing cholangitis and autoimmune pancreatitis: histological assessment of biopsies from Vater's ampulla and the bile duct. *J Gastroenterol Hepatol* 2010; **25**: 1648-1655 [PMID: 20880174 DOI: 10.1111/j.1440-1746.2010.06346.x]

35 **Nakazawa T**, Ohara H, Sano H, Aoki S, Kobayashi S, Okamoto T, Imai H, Nomura T, Joh T, Itoh M. Cholangiography can discriminate sclerosing cholangitis with autoimmune pancreatitis from primary sclerosing cholangitis. *Gastrointest Endosc* 2004; **60**: 937-944 [PMID: 15605009 DOI: 10.1016/S0016-5107(04)02229-1]

36 **Umemura T**, Zen Y, Hamano H, Kawa S, Nakanuma Y, Kiyosawa K. Immunoglobin G4-hepatopathy: association of immunoglobin G4-bearing plasma cells in liver with autoimmune pancreatitis. *Hepatology* 2007; **46**: 463-471 [PMID: 17634963 DOI: 10.1002/hep.21700]

37 **Naitoh I**, Zen Y, Nakazawa T, Ando T, Hayashi K, Okumura F, Miyabe K, Yoshida M, Nojiri S, Kanematsu T, Ohara H, Joh T. Small bile duct involvement in IgG4-related sclerosing cholangitis: liver biopsy and cholangiography correlation. *J Gastroenterol* 2011; **46**: 269-276 [PMID: 20821235 DOI: 10.1007/s00535-010-0319-0]

38 **Kalaitzakis E**, Levy M, Kamisawa T, Johnson GJ, Baron TH, Topazian MD, Takahashi N, Kanno A, Okazaki K, Egawa N, Uchida K, Sheikh K, Amin Z, Shimosegawa T, Sandanayake NS, Church NI, Chapman MH, Pereira SP, Chari S, Webster GJ. Endoscopic retrograde cholangiography does not reliably distinguish IgG4-associated cholangitis from primary sclerosing cholangitis or cholangiocarcinoma. *Clin Gastroenterol Hepatol* 2011; **9**: 800-803.e2 [PMID: 21699807 DOI: 10.1016/j.cgh.2011.05.019]

39 **Loftus EV**, Harewood GC, Loftus CG, Tremaine WJ, Harmsen WS, Zinsmeister AR, Jewell DA, Sandborn WJ. PSC-IBD: a unique form of inflammatory bowel disease associated with primary sclerosing cholangitis. *Gut* 2005; **54**: 91-96 [PMID: 15591511 DOI: 10.1136/gut.2004.046615]

40 **Sano H**, Nakazawa T, Ando T, Hayashi K, Naitoh I, Okumura F, Miyabe K, Yoshida M, Takahashi S, Ohara H, Joh T. Clinical characteristics of inflammatory bowel disease associated with primary sclerosing cholangitis. *J Hepatobiliary Pancreat Sci* 2011; **18**: 154-161 [PMID: 20740366 DOI: 10.1007/s00534-010-0319-8.]

41 **Deshpande V**, Sainani NI, Chung RT, Pratt DS, Mentha G, Rubbia-Brandt L, Lauwers GY. IgG4-associated cholangitis: a comparative histological and immunophenotypic study with primary sclerosing cholangitis on liver biopsy material. *Mod Pathol* 2009; **22**: 1287-1295 [PMID: 19633647 DOI: 10.1038/modpathol.2009.94]

42 **Moon SH**, Kim MH. The role of endoscopy in the diagnosis of autoimmune pancreatitis. *Gastrointest Endosc* 2012; **76**: 645-656 [PMID: 22898422 DOI: 10.1016/j.gie.2012.04.458]

43 **Hyodo N**, Hyodo T. Ultrasonographic evaluation in patients with autoimmune-related pancreatitis. *J Gastroenterol* 2003; **38**: 1155-1161 [PMID: 14714253 DOI: 10.1007/s00535-003-1223-7]

44 **Harada K**, Shimoda S, Kimura Y, Sato Y, Ikeda H, Igarashi S, Ren XS, Sato H, Nakanuma Y. Significance of immunoglobulin G4 (IgG4)-positive cells in extrahepatic cholangiocarcinoma: molecular mechanism of IgG4 reaction in cancer tissue. *Hepatology* 2012; **56**: 157-164 [PMID: 22290731 DOI: 10.1002/hep.25627]

45 **Kubota K**, Kato S, Akiyama T, Yoneda M, Fujita K, Ogawa M, Inamori M, Kobayashi N, Saito S, Kakuta Y, Ohshiro H, Nakajima A. Differentiating sclerosing cholangitis caused by autoimmune pancreatitis and primary sclerosing cholangitis according to endoscopic duodenal papillary features. *Gastrointest Endosc* 2008; **68**: 1204-1208 [PMID: 19028233 DOI: 10.1016/j.gie.2008.08.013]

46 **Sepehr A**, Mino-Kenudson M, Ogawa F, Brugge WR, Deshpande V, Lauwers GY. IgG4+ to IgG+ plasma cells ratio of ampulla can help differentiate autoimmune pancreatitis from other "mass forming" pancreatic lesions. *Am J Surg Pathol* 2008; **32**: 1770-1779 [PMID: 18779730 DOI: 10.1097/PAS.0b013e318185490a]

47 **Itoi T**, Kamisawa T, Igarashi Y, Kawakami H, Yasuda I, Itokawa F, Kishimoto Y, Kuwatani M, Doi S, Hara S, Moriyasu F, Baron TH. The role of peroral video cholangioscopy in patients with IgG4-related sclerosing cholangitis. *J Gastroenterol* 2013; **48**: 504-514 [PMID: 22948487 DOI: 10.1007/s00535-012-0652-6]

48 **Hirano K**, Tada M, Isayama H, Yagioka H, Sasaki T, Kogure H, Nakai Y, Sasahira N, Tsujino T, Yoshida H, Kawabe T, Omata M. Long-term prognosis of autoimmune pancreatitis with and without corticosteroid treatment. *Gut* 2007; **56**: 1719-1724 [PMID: 17525092 DOI: 10.1136/gut.2006.115246]

49 **Topazian M**, Witzig TE, Smyrk TC, Pulido JS, Levy MJ, Kamath PS, Chari ST. Rituximab therapy for refractory biliary strictures in immunoglobulin G4-associated cholangitis. *Clin Gastroenterol Hepatol* 2008; **6**: 364-366 [PMID: 18328441 DOI: 10.1016/j.cgh.2007.12.020]

50 **Nakazawa T**, Ohara H, Ando T, Hayashi K, Naitoh I, Okumura F, Tanaka H, Sano H, Joh T. Clinical course and indications for steroid therapy of sclerosing cholangitis associated with autoimmune pancreatitis. *Hepatogastroenterology* 2009; **56**: 584-588 [PMID: 19621659]

51 **Hirano A**, Nakazawa T, Ohara H, Ando T, Hayashi K, Tanaka H, Naito I, Okumura F, Yokoyama Y, Joh T. Liver atrophy and portal stenosis in two cases of sclerosing cholangitis associated with autoimmune pancreatitis. *Intern Med* 2008; **47**: 1689-1694 [PMID: 18827417 DOI: 10.2169/internalmedicine.47.1192]

52 **Moon SH**, Kim MH, Park DH, Hwang CY, Park SJ, Lee SS, Seo DW, Lee SK. Is a 2-week steroid trial after initial negative investigation for malignancy useful in differentiating autoimmune pancreatitis from pancreatic cancer? A prospective outcome study. *Gut* 2008; **57**: 1704-1712 [PMID: 18583399 DOI: 10.1136/gut.2008.150979]

53 **Tomiyama T**, Uchida K, Matsushita M, Ikeura T, Fukui T, Takaoka M, Nishio A, Okazaki K. Comparison of steroid pulse therapy and conventional oral steroid therapy as initial treatment for autoimmune pancreatitis. *J Gastroenterol* 2011; **46**: 696-704 [PMID: 21188426 DOI: 10.1007/s00535-010-0361-y]

54 **Nakazawa T**, Naitoh I, Ando T, Hayashi K, Okumura F, Miyabe K, Yoshida M, Ohara H, Joh T. A case of advanced-stage sclerosing cholangitis with autoimmune pancreatitis not responsive to steroid therapy. *JOP* 2010; **11**: 58-60 [PMID: 20065555]

55 **Chari ST**, Smyrk TC, Levy MJ, Topazian MD, Takahashi N, Zhang L, Clain JE, Pearson RK, Petersen BT, Vege SS, Farnell MB. Diagnosis of autoimmune pancreatitis: the Mayo Clinic experience. *Clin Gastroenterol Hepatol* 2006; **4**: 1010-106; quiz 934 [PMID: 16843735 DOI: 10.1016/j.cgh.2006.05.017]

P- Reviewers: Kin T, Mizrahi S, Nanashima A
S- Editor: Wen LL **L- Editor:** Kerr C **E- Editor:** Ma S

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v19.i43.7671

World J Gastroenterol 2013 November 21; 19(43): 7671-7679
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Efficacy of switching to telbivudine plus adefovir in suboptimal responders to lamivudine plus adefovir

Hana Park, Jun Yong Park, Seung Up Kim, Do Young Kim, Kwang-Hyub Han, Chae Yoon Chon, Sang Hoon Ahn

Hana Park, Department of Internal Medicine, CHA Bundang Medical Center, CHA University, Seongnam-si 463-712, South Korea
Hana Park, Department of Internal Medicine, Yonsei University College of Medicine, Seoul 120-752, South Korea
Jun Yong Park, Seung Up Kim, Do Young Kim, Chae Yoon Chon, Department of Internal Medicine, Yonsei University College of Medicine, Seoul 120-752, South Korea
Jun Yong Park, Seung Up Kim, Do Young Kim, Chae Yoon Chon, Institute of Gastroenterology, Yonsei University College of Medicine, Liver Cirrhosis Clinical Research Center, Seoul 120-752, South Korea
Kwang-Hyub Han, Sang Hoon Ahn, Department of Internal Medicine, Yonsei University College of Medicine, Seoul 120-752, South Korea
Kwang-Hyub Han, Sang Hoon Ahn, Institute of Gastroenterology, Yonsei University College of Medicine, Seoul 120-752, South Korea
Kwang-Hyub Han, Sang Hoon Ahn, Liver Cirrhosis Clinical Research Center, Seoul 120-752, South Korea
Kwang-Hyub Han, Sang Hoon Ahn, Brain Korea 21 Project for Medical Science, Seoul 120-752, South Korea
Author contributions: Park H, Park JY and Ahn SH designed the research/study; Park H analyzed the data; Park H and Ahn SH performed the study; and Kim SU, Kim DY, Han KH, Chon CY and Ahn SH collected the data; Park JY reviewed the data of study population; Park H wrote the paper.
Supported by A Grant under the Bilateral International Collaborative R and D Program from the Ministry of Knowledge Economy; and the Korea Healthcare Technology R and D Project, Ministry for Health and Welfare, South Korea, No. A102065; the Liver Cirrhosis Clinical Research Center, a grant from the Korea Healthcare Technology R and D project, Ministry of Health and Welfare, South Korea, No. HI10C2020; and a grant to the Bilateral International Collaborative R and D Program from the Ministry of Trade, Industry and Energy, South Korea
Correspondence to: Sang Hoon Ahn, MD, PhD, Department of Internal Medicine, Yonsei University College of Medicine, 150 Shinchon-dong 50 Yonsei-ro, Seodaemun-gu, Seoul 120-752, South Korea. ahnsh@yuhs.ac
Telephone: +82-2-3936884 Fax:+82-2-22281936
Received: June 9, 2013 Revised: September 14, 2013
Accepted: September 16, 2013
Published online: November 21, 2013

Abstract

AIM: To examine the efficacy of telbivudine (LdT) + adefovir (ADV) *vs* continuation of lamivudine (LAM) + ADV in patients with LAM-resistant chronic hepatitis B (CHB) who show a suboptimal response to LAM + ADV.

METHODS: This was a randomized, active-control, open-label, single-center, parallel trial. All eligible patients were enrolled in this study in Severance Hospital, Yonsei University College of Medicine, Seoul, South Korea, between March 2010 and March 2011. Hepatitis Be antigen (HBeAg)-positive CHB patients whose serum hepatitis B virus (HBV) DNA remained detectable despite at least 6 mo of LAM + ADV therapy were included. Enrolled patients were randomized to either switching to LdT (600 mg/d orally) plus ADV (10 mg/d orally) (LdT + ADV group) or to continuation with LAM (100 mg/d orally) plus ADV (10 mg/d orally) (LAM + ADV group), and were followed for 48 wk. One hundred and six patients completed the 48-wk treatment period. Serum HBV DNA, HBeAg status, liver biochemistry and safety were monitored at baseline and week 12, 24, 36 and 48.

RESULTS: The duration of prior LAM + ADV treatment was 18.3 (LdT + ADV) and 14.9 mo (LAM + ADV), respectively (P = 0.131). No difference was seen in baseline serum HBV DNA between the two groups [3.66 (LdT + ADV) *vs* 3.76 (LAM + ADV) $\log_{10}$ IU/mL, P = 0.729]. At week 48, although there was no significant difference in the mean reduction of serum HBV DNA from baseline between LdT + ADV group and LAM + ADV group (-0.81 *vs* -0.47 $\log_{10}$ IU/mL, P = 0.167), more patients in the LdT + ADV group had undetectable HBV DNA levels compared to those in the LAM + ADV group (30.2% *vs* 11.5%, P = 0.019). Three patients with LdT + ADV treatment and 2 patients with LAM + ADV treatment achieved HBeAg loss. The patients in both groups tolerated the treatment well without serious adverse events. The proportion of patients with estimated glomerular filtration rate $\geqslant$ 90 mL/min per 1.73 m^2 in the

LdT + ADV group increased from 49.1% (26/53) at baseline to 58.5% (31/53) at week 48, while that in the LAM + ADV group decreased from 37.7% (20/53) at baseline to 30.2% (16/53) at week 48.

CONCLUSION: The switch to LdT + ADV in suboptimal responders to LAM + ADV showed a significantly higher rate of virologic response at week 48. These results suggest that LdT + ADV could be a therapeutic option for patients who are unable to use enofovir disoproxil fumarate for any reason.

Key words: Chronic hepatitis B; Antiviral resistance; Suboptimal response; Telbivudine; Lamivudine

Core tip: A suboptimal response is common in patients treated with lamivudine (LAM) + adefovir (ADV) combination therapy and it has also become a new challenge for the management of chronic hepatitis B (CHB) patients. We commenced this study with the effect of telbivudine (LdT) + ADV combination therapy as a rescue therapeutic option in LAM-resistant CHB patients with suboptimal response to LAM + ADV. Our results demonstrated that switching from LAM + ADV to LdT + ADV resulted in superior virologic response, renoprotective effect and similar safety profiles at week 48. These results suggest that LdT + ADV could be a therapeutic option for patients who are unable to use enofovir disoproxil fumarate for any reason.

Park H, Park JY, Kim SU, Kim DY, Han KH, Chon CY, Ahn SH. Efficacy of switching to telbivudine plus adefovir in suboptimal responders to lamivudine plus adefovir. *World J Gastroenterol* 2013; 19(43): 7671-7679 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7671.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7671

INTRODUCTION

Worldwide, over 400 million people suffer from chronic hepatitis B (CHB). Patients with CHB have a 15%-40% life-time risk of developing cirrhosis, hepatic decompensation and hepatocellular carcinoma (HCC)[1,2]. High hepatitis B virus (HBV) DNA concentration in serum in patients with CHB is known as an independent risk factor for disease progression to cirrhosis and HCC[2,3]. Therefore, the treatment goal of antiviral therapy for CHB is to achieve complete suppression of viral replication as rapidly as possible[4-6], because prolonged viremia on therapy can lead to a higher risk of future antiviral drug resistance and therapeutic failure as well as disease progression[2,7,8].

Lamivudine (LAM) has been widely used for treatment of CHB since its first approval at 2002. However, a major limitation of LAM is the development of LAM-resistant YMDD-motif mutations in the viral DNA polymerase, the prevalence of which increases progressively to about 70% after 4 years of treatment[9]. In patients resistant to LAM, add-on combination therapy with LAM and adefovir (ADV) has resulted in lower rates of virologic breakthrough and additional development of genotypic resistance than when switching to ADV or entecavir[10,11]. Thus, LAM + ADV combination therapy has been recommended as a rescue therapy in patients with LAM resistant viral strains in many Asian countries, for its considerable effectiveness, lower resistance and affordable price[5].

Unfortunately, a substantial proportion of patients treated with LAM + ADV combination therapy show a suboptimal virologic response[10-12]. Because there has been evidence that this suboptimal response to antiviral therapy might have clinical relevance to higher risk of developing resistance to long-term antiviral treatment, suboptimal response to nucleotide analogues (NAs), in addition to drug resistance, has also become a new challenge for the management of CHB patients[13-15]. However, there is no standard optimal strategy for the management of suboptimal response to NA therapy at present. Many practice guidelines suggest a combination treatment regimen with tenofovir disoproxil fumarate (TDF) which is a NA with a high barrier to resistance as a highly potent rescue therapeutic option[4,6]. TDF, however, remains largely unavailable in Asian countries. Thus, several trials with various combination regimens for these populations have been proceeded[16,17], and the results suggested consistently that combination therapy rather than switching to another drug offers a potentially attractive therapeutic option.

Telbivudine (LdT) is one of the licensed NAs which is structurally related to LAM and highly selective for HBV DNA and inhibits viral DNA synthesis with no effect on human DNA or other viruses[18]. The Gestation Linked to Obesity and Environment (GLOBE), the largest trial in CHB, demonstrated that LdT is superior to LAM for all efficacy measures over 2 years of therapy[19,20]. Another trial showed a superior viral suppressive effect of LdT even to ADV in treatment naïve CHB patients[21]. In addition, LDT + ADV combination treatment showed better outcomes against LAM-resistant HBV than ADV alone[22]. Therefore, LdT is a therapeutic option in LAM-resistant hepatitis B patients with suboptimal response to LAM + ADV combination therapy.

In this study, we directly compared the antiviral efficacy of switching to LdT + ADV combination *vs* LAM + ADV continuation in hepatitis Be antigen (HBeAg) positive LAM-resistant hepatitis B patients who showed suboptimal response to LAM + ADV combination treatment.

MATERIALS AND METHODS

Patients

Patients eligible for this study were men and women, aged over 20 years, positive for serum hepatitis B surface antigen (HBsAg) for at least 6 mo, and positive for HBeAg. Inclusion criteria were confirmed mutations in the

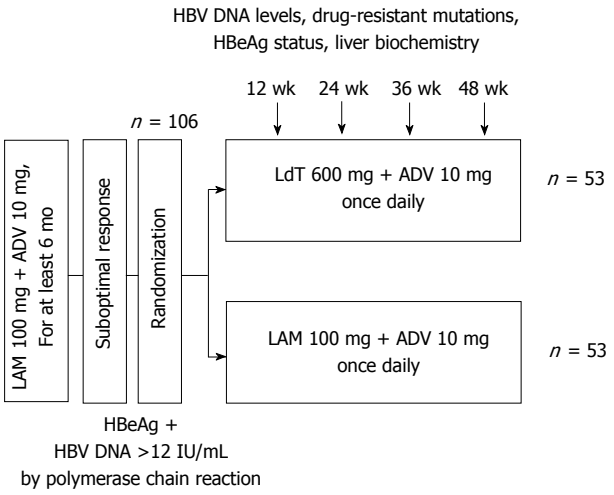


Figure 1 Flow diagram of study participants. LMA: Lamivudine; ADV: Adefovir; LdT: Telbivudine; HBV: High hepatitis B virus; HBeAg: Hepatitis Be antigen.

HBV polymerase gene that confers resistance to LAM (rtM204V/I and/or rtL180M), and serum HBV DNA concentration > 12 IU/mL after combination treatment with LAM (100 mg/d) plus ADV (10 mg/d) for at least 6 mo that was ongoing at the time of randomization. Patients were expected to have well-preserved liver function (Child-Pugh score ⩽ 6) and no history of ascites, variceal bleeding, or encephalopathy.

Patients were excluded if they had previous or current HCC; prior treatment with an antiviral agent other than LAM and/or ADV; coinfection with hepatitis C, hepatitis D, or human immune deficiency virus; concurrent systemic corticosteroids or other immunosuppressive agents; history of alcohol or substance abuse; or other current liver diseases, prior organ transplantation, or a history of malignancy within 3 years.

Study design

This was a randomized, active-control, open-label, single-center, parallel trial. All eligible patients were enrolled in this study in Severance Hospital, Yonsei University College of Medicine, Seoul, Korea, between March 2010 and March 2011. Patients were randomized to either switching to LdT (600 mg/d orally) plus ADV (10 mg/d orally) (LdT + ADV group) or to continuation with LAM (100 mg/d orally) plus ADV (10 mg/d orally) (LAM + ADV group), and were followed for 48 wk. Randomized patients were evaluated at baseline and week 12, 24, 36 and 48. At each visit, hematology, biochemistry, and prothrombin time/international normalized ratio were assessed. HBV DNA level was measured at baseline and week 12, 24, 36 and 48, using a real-time Polymerase Chain Reaction assay (Abbott Laboratories, Chicago, IL) with a linear dynamic detection range of 12 to 1×10^9 IU/mL. Multiplex Restriction fragment mass polymorphism (RFMP) assays of the HBV genome were performed to detect LAM and ADV resistance mutations at baseline and at times as needed[23]. Because over 98% of South Korean patients with CHB have HBV genotype C[24,25], HBV genotype was not determined. HBeAg and anti-HBeAb were assessed at baseline and at week 48, using commercially available enzyme immunoassays (Abbott Laboratories)[26,27]. The upper limit of normal (ULN) alanine aminotransferase (ALT) was defined as 40 IU/L. Occurrences of adverse events were assessed at every visit through week 48.

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and the Guidelines for Good Clinical Practice as well as local regulatory requirements. This study was approved by the Institutional Review Board of Yonsei University of Medical College, and written informed consent was obtained from all patients. This study was registered at ClinicalTrials.gov, number NCT01270165 (http://www.clinicaltrials.gov/ct2/show/NCT01270165).

Study endpoints

The primary endpoint was the proportion of patients in each treatment group who achieved virologic response (serum HBV DNA concentration of < 12 IU/mL) at week 48. Secondary endpoints included mean reduction from baseline in serum HBV DNA concentration at week 48, the proportion of patients with normalized serum ALT levels, HBeAg loss or seroconversion at week 48, and emergence of resistance mutation to drug during study period.

Statistical analysis

The variables were expressed as mean with SD or ranges, or *n* (%), as appropriate. The χ^2 or Fisher's exact test and the Mann-Whitney *U* test were used to compare categorical and continuous variables, respectively. Paired related data were analyzed using the Wilcoxon paired test. A two-sided *P* value < 0.05 was considered to indicate statistical significance. All statistical analyses were performed with SPSS 18.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Baseline characteristics of patients

One hundred and ten patients were screened from March 2010 to March 2011, and 106 were randomized (53 in each group). All patients completed 48 wk of treatment after randomization; thus, data from all 106 patients randomized were available for the intention-to-treat analysis (Figure 1).

Overall baseline characteristics of all patients as well as of each group are shown in Table 1. Twenty-four (22.6%) patients had cirrhosis with well-preserved liver function. The mean (SD) serum HBV DNA levels was 3.71 (1.46) $\log_{10}$ IU/mL. The mean (ranges) duration of LAM + ADV treatment prior to randomization was 17.1 (6-45) mo. At baseline, all patients had LAM resistance mutations, including 27 (25.5%) with rtM204I alone, 1 (0.9%) with rtM204I + rtM204V, 28 (26.4%) with rtM204I + rtL180M, 28 (26.4%) with rtM204V + rtL180M and 22 (20.8%) with rtM204I + rtM204V +

Table 1 Baseline characteristics of patients *n* (%)

Variables	Total (*n* = 106)	LdT + ADV (*n* = 53)	LAM + ADV (*n* = 53)	*P* value
Mean age, yr	46.3 (22-76)	49.0 (23-76)	43.7 (22-73)	0.053
Male	79 (74.5)	42 (79.2)	37 (69.8)	0.265
Liver cirrhosis	24 (22.6)	13 (24.5)	11 (20.8)	0.647
Laboratory results				
AST (IU/L)	29 (7-119)	33 (7-119)	28 (10-92)	0.125
ALT (IU/L)	28 (13-125)	26 (15-84)	29 (13-125)	0.098
Total bilirubin (mg/dL)	0.7 (0.3-1.8)	0.7 (0.3-1.8)	0.8 (0.3-1.6)	0.382
Albumin (g/dL)	4.5 (0.7-5.4)	4.4 (3.4-5.4)	4.6 (0.7-5.1)	0.777
Prothrombin time	1.01 (0.91-1.42)	1.00 (0.91-1.42)	1.02 (0.93-1.24)	0.917
Platelet count ($\times 10^9$/L)	175 (45-293)	175 (67-290)	174 (45-293)	0.610
AFP (ng/mL)	2.87 (0.86-57.54)	2.61 (1.57-11.66)	2.98 (0.86-57.54)	0.030
Mean prior LAM period, mo (range)	32.2 (8-139)	31.5 (8-77)	33.5 (11-139)	0.695
Mean prior LAM + ADV period, mo (range)	17.1 (6-45)	18.3 (6-45)	14.9 (6-39)	0.131
YMDD mutation	106 (100)	53 (100)	53 (100.0)	-
rtM204I alone	27 (25.5)	13 (24.5)	14 (26.4)	0.643
rtM204I + rtM204V	1 (0.9)	1 (1.9)		
rtM204I + rtL180M	28 (26.4)	15 (28.4)	13 (24.5)	
rtM204V + rtL180M	28 (26.4)	12 (22.6)	16 (30.2)	0.501
rtM204I + rtM204V + rtL180M	22 (20.8)	12 (22.6)	10 (18.9)	0.514
eGFR (mL/min per 1.73 m^2)	89.1 (56.1-131.6)	89.8 (59.8-131.6)	85.7 (56.1-123.3)	0.437
Serum HBV DNA (log_{10} IU/mL)				0.729
Mean (SD)	3.71 (1.46)	3.66 (1.65)	3.76 (1.25)	
Median (range)	3.63 (1.32-8.10)	3.34 (1.32-8.10)	3.78 (1.41-5.94)	

Data expressed as mean (SD), mean (range) or median (range). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LAM: Lamivudine; ADV: Adefovire; HBV: High hepatitis B virus; eGFR: Estimated glomerular filtration rate; LdT: Telbivudine.

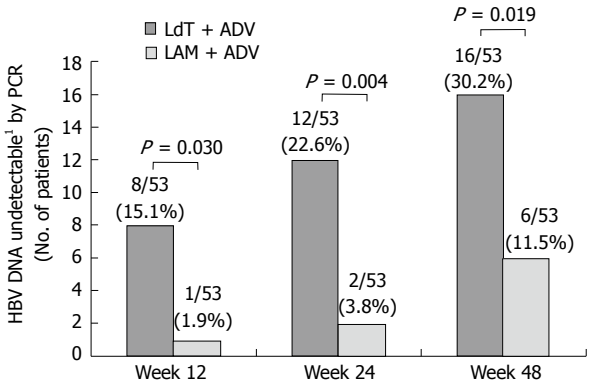


Figure 2 Proportion of patients with undetectable serum hepatitis B virus DNA (< 12 IU/mL) over time. [1]Undetectable < 12 IU/mL. LAM: Lamivudine; ADV: Adefovire; HBV: High hepatitis B virus; LdT: Telbivudine; PCR: Polymerase chain reaction.

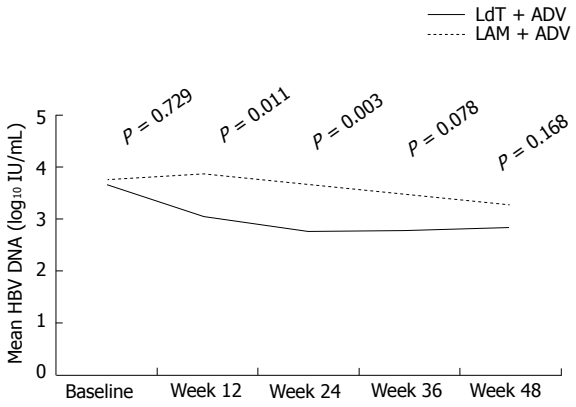


Figure 3 Mean hepatitis B virus DNA levels over time in the two groups. LAM: Lamivudine; ADV: Adefovire; HBV: Hepatitis B virus; LdT: Telbivudine.

rtL180M. There were no genotypic mutations of ADV in all patients at the baseline. Demographic and laboratory characteristics were similar between the two treatment groups, and mean (SD) serum HBV DNA levels in the LdT + ADV group and LAM + ADV group were 3.66 (1.65) log_{10} IU/mL and 3.76 (1.25) log_{10} IU/mL, respectively (P = 0.729). There was no difference in the mean duration of prior LAM treatment as well as that of LAM + ADV treatment prior to randomization between the two groups (prior LAM period, 31.5 mo *vs* 33.5 mo, P = 0.695; LAM + ADV period prior to randomization, 18.3 mo *vs* 14.9 mo, P = 0.131).

Virologic response

The efficacy of treatment in the LdT + ADV and LAM + ADV groups are summarized and compared in Table 2 and Figures 2-4. During treatment, the number of patients who achieved virologic response (serum HBV DNA level of < 12 IU/mL) gradually increased to 16 (30.2%) patients at week 48 in the LdT + ADV group. In contrast, the number of patients with virologic response in the LAM + ADV group was consistently lower than those in the LdT + ADV group from week 12 to week 48, and only 6 (11.5%) patients in the LAM + ADV group showed virologic response at week 48. The primary efficacy endpoint, the proportion of patients who achieved HBV DNA level of < 12 IU/mL at week 48, differed significantly between the two groups (30.2 % *vs* 11.5 %, respectively, P = 0.019) (Figure 2 and Table 2).

Mean (SD) serum HBV DNA level of the LdT +

Table 2 Virologic, serologic and biochemical responses during study periods *n* (%)

Variables	Week 12		*P* value	Week 24		*P* value	Week 48		*P* value
	LdT + ADV	LAM + ADV		LdT + ADV	LAM + ADV		LdT + ADV	LAM + ADV	
Serum HBV DNA, mean (SD) (log_{10} IU/mL)	3.05 (1.51)	3.84 (1.35)	0.011	2.79 (1.52)	3.65 (1.44)	0.003	2.85 (1.73)	3.29 (1.49)	0.168
Reductions in HBV DNA[1], mean (SD) (log_{10} IU/mL)	-0.68 (0.83)	0.07 (0.60)	< 0.001	-0.88 (1.06)	-0.11 (0.85)	< 0.001	-0.81 (1.43)	-0.47 (1.04)	0.167
HBV DNA undetectable[2]	8 (15.1)	1 (1.9)	0.030	12 (22.6)	2 (3.8)	0.004	16 (30.2)	6 (11.5)	0.019
Virologic nonresponders[3]	-	-	-	33 (62.3)	48 (90.6)	0.001	-	-	-
HBsAg loss	0 (0)	0 (0)	-	0 (0)	0 (0)	-	0 (0)	0 (0)	-
HBeAg negativity,	0 (0)	0 (0)	-	2 (3.8)	0 (0)	-	3 (5.7)	2 (3.8)	0.648
Normal range of ALT[4]	39 (73.6)	38 (71.7)	0.828	37 (69.8)	40 (75.5)	0.513	40 (75.5)	38 (71.7)	0.768
ALT normalization[5]	2/14 (14.3)	6/14 (42.9)	0.209	6/14 (42.9)	5/14 (35.7)	0.704	8/14 (57.1)	3/14 (21.4)	0.053

[1]Reduction of hepatitis B virus (HBV) DNA from baseline; [2]Defined serum HBV DNA of < 12 IU/mL; [3]Defined as a < 1 log_{10} IU/mL reduction in serum HBV DNA level from baseline at 24 wk; [4]Upper normal limit of ALT, 40 IU/L; [5]Among patients who have elevated ALT levels at baseline. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; LAM: Lamivudine; ADV: Adefovir. HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis Be antigen.

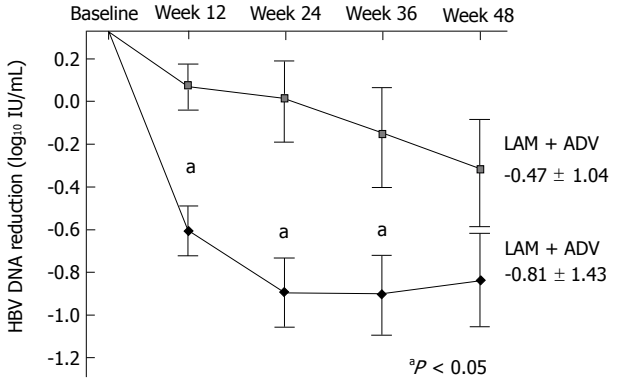


Figure 4 Mean reduction of serum hepatitis B virus DNA levels from baseline. Mean hepatitis B virus (HBV) DNA (log_{10} IU/mL) were plotted over time. Error bars indicate the standard deviation (^aP value < 0.05). LAM: Lamivudine; ADV: Adefovire; LdT: Telbivudine.

ADV group was significantly lower than that of the LAM + ADV group at week 12 and week 24 [3.05 (1.51) log_{10} IU/mL *vs* 3.84 (1.35) log_{10} IU/mL at week 12, P = 0.011; 2.79 (1.52) log_{10} IU/mL *vs* 3.65 (1.44) log_{10} IU/mL at week 24, P = 0.003] (Table 2 and Figure 3). However, there was no statistically significant difference in serum HBV DNA levels between the LdT + ADV group and the LAM + ADV group [2.85 (1.73) log_{10} IU/mL *vs* 3.29 (1.49) log_{10} IU/mL at 48 wk, P = 0.168] (Figure 3).

The mean reduction of serum HBV DNA levels from baseline to week 12 or week 24 was significantly greater in the LdT + ADV than in the LAM + ADV group (-0.68 log_{10} IU/mL *vs* 0.07 log_{10} IU/mL; P < 0.001, -0.88 log_{10} IU/mL *vs* -0.11 log_{10} IU/mL; P < 0.001, respectively) (Figure 4 and Table 2). At week 48, however, there was no significant difference in the mean reduction of serum HBV DNA from baseline between the LdT + ADV group and the LAM + ADV group (-0.81 log_{10} IU/mL *vs* -0.47 log_{10} IU/mL, P = 0.167; Table 2 and Figure 4).

The number of patients with virologic nonresponse, defined as < 1 log_{10} IU/mL reduction in serum HBV DNA level from baseline at week 24, was significantly lower in the LdT + ADV group than in the LAM + ADV group [33 (62.3%) *vs* 48 (90.6%), respectively, P = 0.001] (Table 2). A total of 8 patients experienced virologic breakthrough (≥ 1 log_{10} IU/mL increase in serum HBV DNA from nadir during treatment), 4 patients in the LdT + ADV group and 4 patients in the LAM + ADV group. Most of them (6/8) had poor compliance for taking medication, and there was no new emergence of drug resistance for LAM or ADV in the RFMP examination conducted at the time of virologic breakthrough.

Biochemical and serologic response

The proportion of patients with normal serum ALT levels at week 48 did not differ significantly between the LdT + ADV group and the LAM + ADV group (75.5% *vs* 71.7%, respectively; P = 0.768) (Table 2). Among patients with elevated ALT at baseline, the proportion of patients achieving normalized ALT at week 48 in the LdT + ADV group and LAM + ADV group were 57.1% (8/14) and 21.4% (3/14), respectively, and the difference showed borderline significance between the two groups (P = 0.053) (Table 2).

Three patients (5.7%) in the LdT + ADV group and 2 patients (3.8%) in the LAM + ADV group became HBeAg negative at week 48 (P = 0.648; Table 2). No patient achieved loss of HBsAg during the treatment period.

Safety

The majority of patients in the LdT + ADV and LAM + ADV groups tolerated the treatment well without serious adverse events. No patient required dose reduction or discontinuation of treatment due to an adverse event. No patient experienced ALT flare (> 10 × ULN), increased serum creatinine kinase level of > 150 U/L, or serum phosphorus level of < 1.5 mg/dL during the treatment period. Neither group reported decompensated cirrhosis or hepatocellular carcinoma from baseline to week 48.

No patient was found to have an elevation of creatinine ≥ 0.5 mg/dL. The mean estimated glomerular filtration rate (eGFR) is shown in Figure 5A. Although statistical significance did not exist, eGFR in the LdT + ADV group tended to increase during the treatment pe-

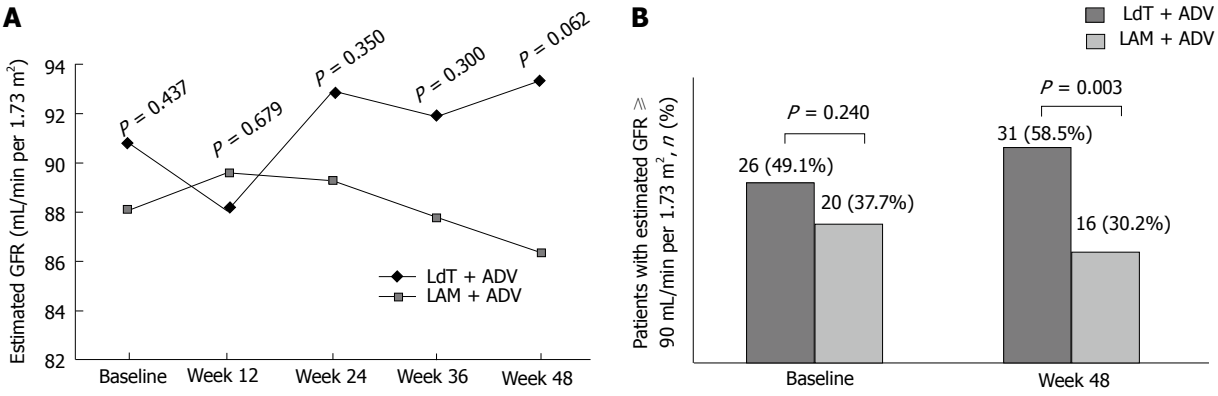


Figure 5 Estimated glomerular filtration rate over time in the two groups (A), and proportion of patients with estimated glomerular filtration rate ⩾ 90 mL/min per 1.73 m² at baseline and week 48 (B). LAM: Lamivudine; ADV: Adefovire; HBV: High hepatitis B virus; LdT: Telbivudine. GFR: Glomerular filtration rate

riod, whereas that in the LAM + ADV group tended to decrease. The proportion of patients with eGFR ⩾ 90 mL/min per 1.73 m² in the LdT + ADV group increased from 49.1% (26/53) at baseline to 58.5% (31/53) at week 48, while that in the LAM + ADV group decreased from 37.7% (20/53) at baseline to 30.2% (16/53) at week 48. The proportion of patients with eGFR ⩾ 90 mL/min per 1.73 m² was significantly higher in the LdT + ADV group than in the LAM + ADV group at week 48 (58.5% *vs* 30.2%; $P = 0.003$) (Figure 5B). Twenty-six percent (7/27) of the patients with baseline eGFR < 90 mL/min per 1.73 m² shifted to eGFR ⩾ 90 mL/min per 1.73 m² after 48 wk of LdT + ADV treatment, as compared to 15.2% (5/33) in the LAM + ADV group ($P = 0.299$).

DISCUSSION

This study is the first study that provides a direct comparison of the antiviral efficacy of LdT + ADV and LAM + ADV in HBeAg-positive LAM resistant CHB patients who have suboptimal response to LAM + ADV. The results of this study show that treatment with LdT + ADV significantly suppressed HBV replication and more patients with LdT + ADV achieved virologic response compared to those with LAM + ADV after 48 wk of treatment. The difference in viral suppressive effect between the two groups was greatest at week 24, which decreased gradually thereafter.

The combination of LAM and ADV has been recommended as a treatment option for patients with LAM resistant CHB[4-6]. Because of the unavailability of TDF in many Asian countries, ADV has been used widely as a combination treatment regimen. However, due to the weak antiviral activity of ADV[28] and poor susceptibility for drug-resistant viral strains, suboptimal response is particularly common in patients who received LAM + ADV[29,30]. Evidence has shown that the persistence of suboptimal response during long-term antiviral treatment is associated with the emergence of multi-drug resistant viral strains, which could result in poorer clinical outcomes[31,32]. Thus, management of a suboptimal response to antiviral therapy has recently been of new concern, and combination with other NAs rather than switching to monotherapy offers a potentially attractive therapeutic option[33,34].

Based on the superior efficacy of LdT over LAM shown in the GLOBE trial[35], a recent study examined switching patients who remained viremic under LAM treatment to LdT and demonstrated that early (⩽ 24 wk) switch to LdT improves virologic outcomes in CHB patients with persistent viral replication under LAM treatment[36]. In addition, previous two independent short-term studies on patients with poor response to ADV monotherapy demonstrated that a higher proportion of patients in the LdT + ADV group achieved a virological response at week 24 than did patients in the LAM + ADV group[37,38]. Based on these prior reports, we conducted this study to investigate the efficacy of switching to LdT + ADV as a substitute therapeutic option for patients who showed a suboptimal response to LAM + ADV combination treatment.

In our study, patients who were switched to LdT + ADV had a superior virologic response at 48 wk compared to those who continued LAM + ADV treatment (30.2% *vs* 11.5%, $P = 0.019$). At 48 wk, the mean serum HBV DNA level was lower and the mean reduction from baseline was greater in the LdT + ADV group than in the LAM + ADV (2.85 $\log_{10}$ IU/mL *vs* 3.29 $\log_{10}$ IU/mL and -0.81 $\log_{10}$ IU/mL *vs* -0.47 $\log_{10}$ IU/mL, respectively), but the differences between the two groups were not statistically significant ($P = 0.168$ and $P = 0.167$, respectively). As described in Table 2, however, differences of both the mean serum HBV DNA levels and the mean reduction of HBV DNA levels from baseline were significant at 12 wk and 24 wk. These results are ascribable to a different rate of decline in serum HBV DNA levels between the two groups. When we analyzed the rate of decline between adjacent time points in the respective treatment groups, we found that there were no statistically significant declines of serum HBV DNA levels as times go by in the LAM + ADV group (Figure 6). Continuing LAM + ADV with suboptimal response offers little antiviral benefit to patients with LAM-resistant HBV and as much as 90.6% of patients who continued on LAM + ADV re-

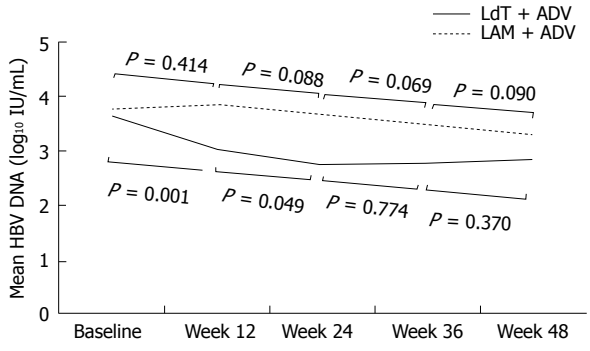


Figure 6 Serial changes of mean serum hepatitis B virus DNA levels. Serum hepatitis B virus DNA levels decreased significantly not only from baseline to 12 wk but also from 12 wk to 24 wk in the LdT + ADV group. LAM: Lamivudine; ADV: Adefovire; HBV: High hepatitis B virus; LdT: Telbivudine.

mained as virologic non-responders (defined as < 1 $\log_{10}$ IU/mL reduction in baseline serum HBV DNA level at 24 wk) at week 24. In contrast, serum HBV DNA levels decreased significantly not only from baseline to 12 wk but also from 12 wk to 24 wk in the LdT + ADV group ($P < 0.001$ and $P = 0.049$, respectively) (Figure 6). This suggests that the viral suppressive effect emerged in the LdT + ADV group particularly during the early treatment period.

Considering the significantly decreased serum HBV DNA level in the LdT + ADV group during the early treatment period, we speculated the good virologic response in this group may be related to the effect of LdT, because evidence in treatment-naïve patients demonstrates that LdT could significantly increase the rate of virologic response compared to LAM as well as ADV[19,39,40]. However, the rate of serum HBV DNA level decline in the LdT + ADV group decreased and became dull at the later part of the study period, which is from week 24 to week 48. It might be correlated to diminished susceptibility to NAs and generally unsatisfying clinical outcomes in a pretreated population with drug-resistant HBV compared to a treatment-naïve population[41-43]. Another possible explanation is that the emergence of ADV-resistant HBV strains following suboptimal response to LAM + ADV might attenuate the superior viral suppressive effect of LdT to that of LAM in these study patients. This is supported by a recent study which reported no differences in virologic and biochemical responses in the comparison of two treatments, LdT + ADV and LAM + ADV, in CHB patients with suboptimal response to ADV monotherapy[44].

HBeAg loss is the key goal of antiviral therapy for HBeAg-positive CHB patients, which indicates good prognosis, including lower rates of cirrhosis and slower disease progression[5,6,45,46]. In our study, we reported quite low rates of HBeAg loss, with 5.7% in the LdT + ADV group and 3.8% in the LAM + ADV group, suggesting that this pretreated population is particularly refractory to serologic response. It has been reported that HBeAg loss is less common in patients with LAM-resistant mutation than in those with wild-type HBV, regardless of the adequate rescue therapy[41-43].

Both treatments were well tolerated and showed similar safety profiles. Patients who switched from LAM + ADV to LdT + ADV did not experience any additional spectrum of adverse effects. Interestingly, we found that the patients in the LdT + ADV group showed a favorable effect of improved renal function compared to those in the LAM + ADV group during the treatment period. Although the mechanism has not been clarified, there have been several reports that LdT treatment is associated with renoprotective effects in patients with CHB[47,48]. Considering the risk of renal impairment of ADV, the renoprotective effect of LdT could be complementary in patients who were treated with ADV for a long term period.

There are some limitations in this study. First, this prospective study has small sample size and a potential bias. Relatively short follow-up duration was another limitation. Thus, further well controlled studies with sufficient size and longer duration of follow-up are needed.

In conclusion, this trial demonstrated that switching from LAM + ADV to LdT + ADV in LAM-resistant CHB patients with suboptimal response resulted in superior virologic response, renoprotective effect and similar safety profiles at week 48. These results suggest that CHB patients with LAM-resistant HBV and suboptimal response to LAM + ADV treatment should be considered for switching to other combination regimens using more potent drugs. LdT + ADV could be a therapeutic option for patients who are unable to use TDF for any reason. However, a stronger rescue combination therapy should be investigated in this population.

ACKNOWLEDGMENTS

The authors are grateful to Dong-Su Jang (Medical Illustrator, Medical Research Support Section, Yonsei University College of Medicine, Seoul, South Korea) for his help with the figures.

COMMENTS

Background

A substantial proportion of patients treated with lamivudine (LAM) + adefovir (ADV) combination therapy show a suboptimal virologic response. Because there has been evidence that this suboptimal response to antiviral therapy might have clinical relevance to higher risk of developing resistance to long-term antiviral treatment, suboptimal response to nucleotide analogues, in addition to drug resistance, has also become a new challenge for the management of chronic hepatitis B (CHB) patients. However, there is no standard optimal strategy for the management of suboptimal response to nucleotide analogue therapy at present.

Research frontiers

This study is the first study that provides a direct comparison of the antiviral efficacy of telbivudine (LdT) + ADV and LAM + ADV in hepatitis Be antigen-positive LAM resistant CHB patients who have suboptimal response to LAM + ADV.

Innovations and breakthroughs

Our results demonstrated that switching from LAM + ADV to LdT + ADV in LAM-resistant CHB patients with suboptimal response resulted in superior virologic

response, renoprotective effect and similar safety profiles at week 48.

Applications

From our study, it was suggested that LdT + ADV could be a therapeutic option for patients who are unable to use tenofovir disoproxil fumarate for any reason.

Peer review

The authors described a comparison of combination therapy of telbivudine plus adefovir vs lamivudine plus adefovir. This is the first comparison study about these 2 different combination therapies. This information is very important for future antiviral therapy of chronic B hepatitis. Furthermore, the study design is well organized and data is analyzed very well.

REFERENCES

1 **Lavanchy D**. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepat* 2004; **11**: 97-107 [PMID: 14996343]

2 **Chen CJ**, Yang HI, Su J, Jen CL, You SL, Lu SN, Huang GT, Iloeje UH. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA* 2006; **295**: 65-73 [PMID: 16391218 DOI: 10.1001/jama.295.1.65]

3 **Iloeje UH**, Yang HI, Su J, Jen CL, You SL, Chen CJ. Predicting cirrhosis risk based on the level of circulating hepatitis B viral load. *Gastroenterology* 2006; **130**: 678-686 [PMID: 16530509 DOI: 10.1053/j.gastro.2005.11.016]

4 **European Association For The Study Of The Liver.** EASL Clinical Practice Guidelines: management of chronic hepatitis B. *J Hepatol* 2009; **50**: 227-242 [PMID: 19054588 DOI: 10.1016/j.jhep.2008.10.001]

5 **Liaw YF**, Leung N, Kao JH, Piratvisuth T, Gane E, Han KH, Guan R, Lau GK, Locarnini S. Asian-Pacific consensus statement on the management of chronic hepatitis B: a 2008 update. *Hepatol Int* 2008; **2**: 263-283 [PMID: 19669255 DOI: 10.1007/s12072-008-9080-3]

6 **Lok AS**, McMahon BJ. Chronic hepatitis B: update 2009. *Hepatology* 2009; **50**: 661-662 [PMID: 19714720 DOI: 10.1002/hep.23190]

7 **Yuen MF**, Tanaka Y, Fong DY, Fung J, Wong DK, Yuen JC, But DY, Chan AO, Wong BC, Mizokami M, Lai CL. Independent risk factors and predictive score for the development of hepatocellular carcinoma in chronic hepatitis B. *J Hepatol* 2009; **50**: 80-88 [PMID: 18977053 DOI: 10.1016/j.jhep.2008.07.023]

8 **Gish RG**. Improving outcomes for patients with chronic hepatitis B. *Curr Gastroenterol Rep* 2007; **9**: 14-22 [PMID: 17335673]

9 **Lai CL**, Dienstag J, Schiff E, Leung NW, Atkins M, Hunt C, Brown N, Woessner M, Boehme R, Condreay L. Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. *Clin Infect Dis* 2003; **36**: 687-696 [PMID: 12627352 DOI: 10.1086/368083]

10 **Lampertico P**, Viganò M, Manenti E, Iavarone M, Sablon E, Colombo M. Low resistance to adefovir combined with lamivudine: a 3-year study of 145 lamivudine-resistant hepatitis B patients. *Gastroenterology* 2007; **133**: 1445-1451 [PMID: 17983801 DOI: 10.1053/j.gastro.2007.08.079]

11 **Rapti I**, Dimou E, Mitsoula P, Hadziyannis SJ. Adding-on versus switching-to adefovir therapy in lamivudine-resistant HBeAg-negative chronic hepatitis B. *Hepatology* 2007; **45**: 307-313 [PMID: 17256746 DOI: 10.1002/hep.21534]

12 **Heo NY**, Lim YS, Lee HC, Chung YH, Lee YS, Suh DJ. Lamivudine plus adefovir or entecavir for patients with chronic hepatitis B resistant to lamivudine and adefovir. *J Hepatol* 2010; **53**: 449-454 [PMID: 20646776 DOI: 10.1016/j.jhep.2010.03.020]

13 **Lampertico P**. Partial virological response to nucleos(t)ide analogues in naïve patients with chronic hepatitis B: From guidelines to field practice. *J Hepatol* 2009; **50**: 644-647 [PMID: 19231017 DOI: 10.1016/j.jhep.2009.01.007]

14 **Santantonio T**, Fasano M, Durantel S, Barraud L, Heichen M, Guastadisegni A, Pastore G, Zoulim F. Adefovir dipivoxil resistance patterns in patients with lamivudine-resistant chronic hepatitis B. *Antivir Ther* 2009; **14**: 557-565 [PMID: 19578241]

15 **Gallego A**, Sheldon J, García-Samaniego J, Margall N, Romero M, Hornillos P, Soriano V, Enrí quez J. Evaluation of initial virological response to adefovir and development of adefovir-resistant mutations in patients with chronic hepatitis B. *J Viral Hepat* 2008; **15**: 392-398 [PMID: 18221300 DOI: 10.1111/j.1365-2893.2007.00966.x]

16 **Xing J**, Han T, Liu L, Li Y, Li J, Li Y, Xiao SX. Entecavir 1.0mg monotherapy or entecavir plus adefovir dipivoxil for patients with lamivudine-resistant chronic hepatitis B had suboptimal response to lamivudine plus adefovir dipivoxil. *Zhonghua Ganzangbing Zazhi* 2011; **19**: 828-832 [PMID: 22433304]

17 **Lim YS**, Lee JY, Lee D, Shim JH, Lee HC, Lee YS, Suh DJ. Randomized trial of entecavir plus adefovir in patients with lamivudine-resistant chronic hepatitis B who show suboptimal response to lamivudine plus adefovir. *Antimicrob Agents Chemother* 2012; **56**: 2941-2947 [PMID: 22430972 DOI: 10.1128/aac.00338-12]

18 **Bryant ML**, Bridges EG, Placidi L, Faraj A, Loi AG, Pierra C, Dukhan D, Gosselin G, Imbach JL, Hernandez B, Juodawlkis A, Tennant B, Korba B, Cote P, Marion P, Cretton-Scott E, Schinazi RF, Sommadossi JP. Antiviral L-nucleosides specific for hepatitis B virus infection. *Antimicrob Agents Chemother* 2001; **45**: 229-235 [PMID: 11120971 DOI: 10.1128/aac.45.1.229-235.2001]

19 **Lai CL**, Gane E, Liaw YF, Hsu CW, Thongsawat S, Wang Y, Chen Y, Heathcote EJ, Rasenack J, Bzowej N, Naoumov NV, Di Bisceglie AM, Zeuzem S, Moon YM, Goodman Z, Chao G, Constance BF, Brown NA. Telbivudine versus lamivudine in patients with chronic hepatitis B. *N Engl J Med* 2007; **357**: 2576-2588 [PMID: 18094378 DOI: 10.1056/NEJMoa066422]

20 **Keeffe EB**, Zeuzem S, Koff RS, Dieterich DT, Esteban-Mur R, Gane EJ, Jacobson IM, Lim SG, Naoumov N, Marcellin P, Piratvisuth T, Zoulim F. Report of an international workshop: Roadmap for management of patients receiving oral therapy for chronic hepatitis B. *Clin Gastroenterol Hepatol* 2007; **5**: 890-897 [PMID: 17632041 DOI: 10.1016/j.cgh.2007.05.004]

21 **Chan HL**, Heathcote EJ, Marcellin P, Lai CL, Cho M, Moon YM, Chao YC, Myers RP, Minuk GY, Jeffers L, Sievert W, Bzowej N, Harb G, Kaiser R, Qiao XJ, Brown NA. Treatment of hepatitis B e antigen positive chronic hepatitis with telbivudine or adefovir: a randomized trial. *Ann Intern Med* 2007; **147**: 745-754 [PMID: 17909201]

22 **Ahn SH,** Kweon YO, Paik SW, Sohn JH, Lee KS, Kim DJ, Piratvisuth T, Yuen MF, Chutaputti A, Chao YC, Trylesinski A, Avila C. Telbivudine in combination with adefovir versus adefovir monotherapy in HBeAg-positive, lamivudine-resistant chronic hepatitis B. *Hepatol Int* 2011 Oct 12; Epub ahead of print [PMID: 21989925 DOI: 10.1007/s12072-011-9314-7]

23 **Han KH**, Hong SP, Choi SH, Shin SK, Cho SW, Ahn SH, Hahn JS, Kim SO. Comparison of multiplex restriction fragment mass polymorphism and sequencing analyses for detecting entecavir resistance in chronic hepatitis B. *Antivir Ther* 2011; **16**: 77-87 [PMID: 21311111 DOI: 10.3851/imp1702]

24 **Bae SH**, Yoon SK, Jang JW, Kim CW, Nam SW, Choi JY, Kim BS, Park YM, Suzuki S, Sugauchi F, Mizokami M. Hepatitis B virus genotype C prevails among chronic carriers of the virus in Korea. *J Korean Med Sci* 2005; **20**: 816-820 [PMID: 16224156]

25 **Kim BK**, Revill PA, Ahn SH. HBV genotypes: relevance to natural history, pathogenesis and treatment of chronic hepatitis B. *Antivir Ther* 2011; **16**: 1169-1186 [PMID: 22155900 DOI: 10.3851/imp1982]

26 **Chon YE**, Kim SU, Lee CK, Heo J, Kim JK, Yoon KT, Cho

M, Lee KS, Kim DH, Choi EH, Park JY, Kim do Y, Chon CY, Han KH, Ahn SH. Partial virological response to entecavir in treatment-naive patients with chronic hepatitis B. *Antivir Ther* 2011; **16**: 469-477 [PMID: 21685534 DOI: 10.3851/imp1772]

27 **Lee JM**, Ahn SH, Kim HS, Park H, Chang HY, Kim do Y, Hwang SG, Rim KS, Chon CY, Han KH, Park JY. Quantitative hepatitis B surface antigen and hepatitis B e antigen titers in prediction of treatment response to entecavir. *Hepatology* 2011; **53**: 1486-1493 [PMID: 21520167 DOI: 10.1002/hep.24221]

28 **Marcellin P**, Heathcote EJ, Buti M, Gane E, de Man RA, Krastev Z, Germanidis G, Lee SS, Flisiak R, Kaita K, Manns M, Kotzev I, Tchernev K, Buggisch P, Weilert F, Kurdas OO, Shiffman ML, Trinh H, Washington MK, Sorbel J, Anderson J, Snow-Lampart A, Mondou E, Quinn J, Rousseau F. Tenofovir disoproxil fumarate versus adefovir dipivoxil for chronic hepatitis B. *N Engl J Med* 2008; **359**: 2442-2455 [PMID: 19052126 DOI: 10.1056/NEJMoa0802878]

29 **Lee JM**, Park JY, Kim do Y, Nguyen T, Hong SP, Kim SO, Chon CY, Han KH, Ahn SH. Long-term adefovir dipivoxil monotherapy for up to 5 years in lamivudine-resistant chronic hepatitis B. *Antivir Ther* 2010; **15**: 235-241 [PMID: 20386079 DOI: 10.3851/imp1510]

30 **Ryu HJ**, Lee JM, Ahn SH, Kim do Y, Lee MH, Han KH, Chon CY, Park JY. Efficacy of adefovir add-on lamivudine rescue therapy compared with switching to entecavir monotherapy in patients with lamivudine-resistant chronic hepatitis B. *J Med Virol* 2010; **82**: 1835-1842 [PMID: 20872709 DOI: 10.1002/jmv.21898]

31 **Ghany MG**, Doo EC. Antiviral resistance and hepatitis B therapy. *Hepatology* 2009; **49**: S174-S184 [PMID: 19399794 DOI: 10.1002/hep.22900]

32 **Locarnini S**. Molecular virology and the development of resistant mutants: implications for therapy. *Semin Liver Dis* 2005; **25** Suppl 1: 9-19 [PMID: 16103977 DOI: 10.1055/s-2005-915645]

33 **Reijnders JG**, Pas SD, Schutten M, de Man RA, Janssen HL. Entecavir shows limited efficacy in HBeAg-positive hepatitis B patients with a partial virologic response to adefovir therapy. *J Hepatol* 2009; **50**: 674-683 [PMID: 19231002 DOI: 10.1016/j.jhep.2008.10.033]

34 **Brunetto MR**, Lok AS. New approaches to optimize treatment responses in chronic hepatitis B. *Antivir Ther* 2010; **15** Suppl 3: 61-68 [PMID: 21041905 DOI: 10.3851/imp1625]

35 **Liaw YF**, Gane E, Leung N, Zeuzem S, Wang Y, Lai CL, Heathcote EJ, Manns M, Bzowej N, Niu J, Han SH, Hwang SG, Cakaloglu Y, Tong MJ, Papatheodoridis G, Chen Y, Brown NA, Albanis E, Galil K, Naoumov NV. 2-Year GLOBE trial results: telbivudine Is superior to lamivudine in patients with chronic hepatitis B. *Gastroenterology* 2009; **136**: 486-495 [PMID: 19027013 DOI: 10.1053/j.gastro.2008.10.026]

36 **Safadi R**, Xie Q, Chen Y, Yin YK, Wei L, Hwang SG, Zuckerman E, Jia JD, Lopez P. Efficacy of switching to telbivudine in chronic hepatitis B patients treated previously with lamivudine. *Liver Int* 2011; **31**: 667-675 [PMID: 21040410 DOI: 10.1111/j.1478-3231.2010.02360.x]

37 **Wang LC**, Chen EQ, Cao J, Liu L, Wang JR, Lei BJ, Tang H. Combination of Lamivudine and adefovir therapy in HBeAg-positive chronic hepatitis B patients with poor response to adefovir monotherapy. *J Viral Hepat* 2010; **17**: 178-184 [PMID: 19656287 DOI: 10.1111/j.1365-2893.2009.01164.x]

38 **Chen EQ ZT,** Tang H. Combination of telbivudine and adefovir dipivoxil therapy in chronic hepatitis B patients with poor response to adefovir dipivoxil monotherapy. *INT J INFECT DIS* 2010; **14** Suppl 2: S18 [DOI: 10.1016/S1201-9712(10)60001-X]

39 **McKeage K**, Keam SJ. Telbivudine: a review of its use in compensated chronic hepatitis B. *Drugs* 2010; **70**: 1857-1883 [PMID: 20836578 DOI: 10.2165/11204330-000000000-00000]

40 **Hartwell D**, Jones J, Harris P, Cooper K. Telbivudine for the treatment of chronic hepatitis B infection. *Health Technol Assess* 2009; **13** Suppl **3**: 23-30 [PMID: 19846025 DOI: 10.3310/hta13suppl3/04]

41 **Chang TT**, Gish RG, Hadziyannis SJ, Cianciara J, Rizzetto M, Schiff ER, Pastore G, Bacon BR, Poynard T, Joshi S, Kleszczewski KS, Thiry A, Rose RE, Colonno RJ, Hindes RG. A dose-ranging study of the efficacy and tolerability of entecavir in Lamivudine-refractory chronic hepatitis B patients. *Gastroenterology* 2005; **129**: 1198-1209 [PMID: 16230074 DOI: 10.1053/j.gastro.2005.06.055]

42 **Peters MG**, Hann Hw Hw, Martin P, Heathcote EJ, Buggisch P, Rubin R, Bourliere M, Kowdley K, Trepo C, Gray Df Df, Sullivan M, Kleber K, Ebrahimi R, Xiong S, Brosgart CL. Adefovir dipivoxil alone or in combination with lamivudine in patients with lamivudine-resistant chronic hepatitis B. *Gastroenterology* 2004; **126**: 91-101 [PMID: 14699491]

43 **Sherman M**, Yurdaydin C, Sollano J, Silva M, Liaw YF, Cianciara J, Boron-Kaczmarska A, Martin P, Goodman Z, Colonno R, Cross A, Denisky G, Kreter B, Hindes R. Entecavir for treatment of lamivudine-refractory, HBeAg-positive chronic hepatitis B. *Gastroenterology* 2006; **130**: 2039-2049 [PMID: 16762627 DOI: 10.1053/j.gastro.2006.04.007]

44 **Chen EQ**, Zhou TY, Bai L, Wang JR, Yan LB, Liang LB, Tang H. Lamivudine plus adefovir or telbivudine plus adefovir for chronic hepatitis B patients with suboptimal response to adefovir. *Antivir Ther* 2012; **17**: 973-979 [PMID: 22728692 DOI: 10.3851/imp2190]

45 **Liaw YF**. HBeAg seroconversion as an important end point in the treatment of chronic hepatitis B. *Hepatol Int* 2009; **3**: 425-433 [PMID: 19669245 DOI: 10.1007/s12072-009-9140-3]

46 **Ahn SH**, Chan HL, Chen PJ, Cheng J, Goenka MK, Hou J, Lim SG, Omata M, Piratvisuth T, Xie Q, Yim HJ, Yuen MF. Chronic hepatitis B: whom to treat and for how long? Propositions, challenges, and future directions. *Hepatol Int* 2010; **4**: 386-395 [PMID: 20305758 DOI: 10.1007/s12072-010-9163-9]

47 **Li X**, Zhong C, Yang S, Fan R, Peng J, Guo Y, Sun J, Hou J. [Influence of adefovir dipivoxil or telbivudine monotherapy on renal function of patients with chronic hepatitis B]. *Nanfang Yike Daxue Xuebao* 2012; **32**: 826-829 [PMID: 22699063]

48 Gane E, Deray G, Piratvisuth T, Chan HLY, Jia J, Ren H, Rasenack J, Manns M, Amarapurkar D, Dong Y, Trylesinski A. Renal function of chronic hepatitis B (CHB) patients improves with telbivudine treatment[R]. Thailand, Bangkok: APASL 2011

P- Reviewer: Yoshida S **S- Editor:** Wen LL
L- Editor: O'Neill M **E- Editor:** Wu HL

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v19.i43.7680

World J Gastroenterol 2013 November 21; 19(43): 7680-7695
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

8-bromo-7-methoxychrysin inhibits properties of liver cancer stem cells *via* downregulation of β-catenin

Mei-Fang Quan, Li-Hong Xiao, Zhi-Hong Liu, Hui Guo, Kai-Qun Ren, Fei Liu, Jian-Guo Cao, Xi-Yun Deng

Mei-Fang Quan, Li-Hong Xiao, Kai-Qun Ren, Fei Liu, Jian-Guo Cao, Xi-Yun Deng, Medical College, Hunan Normal University, Changsha 410013, Hunan Province, China
Zhi-Hong Liu, Hui Guo, Department of pathology, Hunan Provincial Tumor Hospital, Changsha 410013, Hunan Province, China
Author contributions: Quan MF and Xiao LH contributed equally to this work; Quan MF, Xiao LH, Liu ZH, Guo H, Ren KQ and Liu F performed the majority of experiments; Cao JG designed the study and wrote the manuscript; Deng XY participated in the study and helped to draft the manuscript.
Supported by National Natural Science Foundation of China, No. 81172375; and Scientific Research Fund of Hunan Normal University, No. 81105
Correspondence to: Jian-Guo Cao, Professor, Medical College, Hunan Normal University, Yuelu District, Changsha 410013, Hunan Province, China. caojianguo2005@126.com
Telephone: +86-731-88912434 Fax: +86-731-88912417
Received: May 22, 2013 Revised: September 13, 2013
Accepted: September 16, 2013
Published online: November 21, 2013

Abstract

AIM: To evaluate whether 8-bromo-7-methoxychrysin (BrMC), a synthetic analogue of chrysin, inhibits the properties of cancer stem cells derived from the human liver cancer MHCC97 cell line and to determine the potential mechanisms.

METHODS: $CD133^+$ cells were sorted from the MHCC97 cell line by magnetic activated cell sorting, and amplified in stem cell-conditioned medium to obtain the enriched $CD133^+$ sphere forming cells (SFCs). The stem cell properties of $CD133^+$ SFCs were validated by the tumorsphere formation assay *in vitro* and the xenograft nude mouse model *in vivo*, and termed liver cancer stem cells (LCSCs). The effects of BrMC on LCSCs *in vitro* were evaluated by MTT assay, tumorsphere formation assay and transwell chamber assay. The effects of BrMC on LCSCs *in vivo* were determined using a primary and secondary xenograft model in Balb/c-nu mice. Expressions of the stem cell markers, epithelial-mesenchymal transition (EMT) markers and β-catenin protein were analyzed by western blotting or immunohistochemical analysis.

RESULTS: $CD133^+$ SFCs exhibited stem-like cell properties of tumorsphere formation and tumorigenesis capacity in contrast to the parental MHCC97 cells. We found that BrMC preferentially inhibited proliferation and self-renewal of LCSCs ($P < 0.05$). Furthermore, BrMC significantly suppressed EMT and invasion of LCSCs. Moreover, BrMC could efficaciously eliminate LCSCs *in vivo*. Interestingly, we showed that BrMC decreased the expression of β-catenin in LCSCs. Silencing of β-catenin by small interfering RNA could synergize the inhibition of self-renewal of LCSCs induced by BrMC, while Wnt3a treatment antagonized the inhibitory effects of BrMC.

CONCLUSION: BrMC can inhibit the functions and characteristics of LCSCs derived from the liver cancer MHCC97 cell line through downregulation of β-catenin expression.

Key words: Liver cancer; Cancer stem cell; 8-bromo-7-methoxychrysin; Self-renewal; β-catenin

Core tip: We successfully obtained liver cancer stem cells (LCSCs) from the liver cancer MHCC97 cell line by employing the combination of magnetic activated cell sorting and tumorsphere culture. We showed for the first time that 8-bromo-7-methoxychrysin (BrMC), a synthetic analogue of chrysin, could preferentially inhibit proliferation and self-renewal, suppress epithelial-mesenchymal transition and invasion of LCSCs, and further eradicate LCSCs *in vivo*. The results of this study support the use of BrMC for liver cancer chemopreven-

tion or chemotherapy.

Quan MF, Xiao LH, Liu ZH, Guo H, Ren KQ, Liu F, Cao JG, Deng XY. 8-bromo-7-methoxychrysin inhibits properties of liver cancer stem cells *via* downregulation of β-catenin. *World J Gastroenterol* 2013; 19(43): 7680-7695 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7680.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7680

INTRODUCTION

Human liver cancer is the fifth most common cancer in the world and the third leading cause of cancer-related death[1,2]. Although surgery, liver transplantation or chemotherapy offers the possibility of prolonged survival for liver cancer patients, mortality still remains high, largely due to recurrence and drug-resistance[3,4]. According to the cancer stem cell hypothesis, this is thought to be due to the survival of a population of chemoresistant cells within the tumor, the cancer stem cells (CSCs) in liver cancer, that are able to regenerate the tumor following chemotherapy[5]. However, most currently available therapeutic approaches, including chemotherapy and radiotherapy, lack the ability to effectively kill these CSCs, which may eventually lead to the disease relapse and metastasis[6,7]. A number of previous studies have suggested that CD133, originally identified as a hematopoietic stem cell marker, could be used to isolate liver cancer stem cells (LCSCs) from human liver cancer cell lines, xenograft tumors and primary liver cancer specimens[8-13]. These $CD133^+$ liver cancer cells possess many stem cell properties, including extensive proliferation, self-renewal, and differentiation into the bulk of cancer cells. Thus, this minor subpopulation of $CD133^+$ LCSCs may contribute to the high recurrence rate of liver cancer. Therefore, the identification of a compound that can target LCSCs is one of the main steps in improving overall survival of liver cancer patients.

More recently, a number of studies have found that several dietary compounds can directly or indirectly inhibit cancer stem cell self-renewal pathways[14]. For example, natural flavonoid, genistein and a synthetic derivative of daidzein, N-t-boc-daidzein, have been reported to possess inhibitory activity against prostate and epithelial ovarian CSCs, respectively[15,16]. Chrysin (5,7-dihydroxyflavone), a naturally widely distributed flavonoid, has been shown to possess promising effects on the inhibition of proliferation and induction of apoptosis in a variety of cancer cells[17]. 8-bromo-7-methoxychrysin (BrMC) is a synthetic derivative of chrysin, and our previous study demonstrated the effect of BrMC in inhibiting proliferation and induction of apoptosis in colon, gastric and liver cancer cells was stronger than that of chrysin[18-21].

In this study, we investigated the inhibitory effects of BrMC on the characteristics of LCSCs. We showed for the first time that BrMC was able to inhibit cancer stem cell-like properties of LCSCs and eliminate LCSCs *in vivo*. We also found that BrMC significantly decreased β-catenin expression in LCSCs and knockdown of β-catenin expression could synergize the inhibition of self-renewal of LCSCs induced by BrMC. Together, our results indicated that the downregulation of β-catenin expression appeared to contribute to the inhibitory effects of BrMC on the properties of LCSCs.

MATERIALS AND METHODS

Cell culture and reagents

The human liver cancer MHCC97 cell line was purchased from Fuxiang Biotechnology Co., Ltd. (Shanghai, China). MHCC97 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen Life Technologies, Carlsbad, CA, United States) in an incubator containing 5% CO_2 at 37 ℃. Wnt3a-conditioned medium was prepared as described by Willert *et al*[22]. BrMC was synthesized as described previously[18]. MTT was purchased from Sigma (St. Louis, MO, United States). Fetal bovine serum was from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). Trypsin and DMSO were from Amersco Company (Solon, OH, United States). Antibodies used in this study were as follows: rabbit polyclonal antibodies against ZO-1 (Abcam, Cambridge, MA, United States), mouse monoclonal antibodies against N-cadherin (Upstate Co., Lake Placid, NY, United States), Vimentin (Neo Markers, Fremont, CA, United States), E-cadherin (BD Transduction Labs, Lexington, KY, United States), β-catenin and CD44 (Cell Signaling Technology Inc., Danvers, MA, United States), β-actin (Sigma Chemical Co., St Louis, MO, United States), and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, United States).

Cell sorting and flow cytometry

Cell sorting was performed on MHCC97 cells using the cell surface marker $CD133^+$ with magnetic activated cell sorting (MACS) separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Cells were trypsinized and washed with PBS, and suspended in Phosphate buffered saline (PBS) containing 0.5% Bovine Serum Albumin (BSA). 100 μL Fc receptor (FCR) Blocking Reagent (anti-CD133 antibody) and 100 μL CD133-conjugated MicroBeads (AC133, Cell Isolation Kit, Miltenyi Biotec) per 10^8 cells were subsequently added to the sample and incubated in parallel for 30 min on ice. After washing the cells, CD133 positive and negative fractions were each isolated through MACS separation columns. The quality of sorting was controlled by flow cytometry analysis for CD133 expression using PE-conjugated anti-human CD133 antibody and isotype control mouse IgG2b-PE (Biolegend, San Diego, CA, United States). The single cell suspension was cultured in stem cell-conditioned medium (DMEM/F12

medium supplemented with 1 × B27, 20 ng/mL EGF, 20 ng/mL bFGF, 0.4% BSA, 4 μg/mL Insulin, 100 U/mL penicillin and 100 μg/mL streptomycin; Invitrogen) for the following assays.

Tumorsphere culture

Single-cell suspensions were suspended at a density of 2000 cells/mL in stem cell-conditioned medium and seeded into ultralow attachment 24-well plates (Corning, NY, United States). When the diameter of the spheroid reached 50 μm, suspension cultures were passaged every 6 d. Colonies were counted at 10 different views under a microscope (Nikon, Japan). The volume of the spheroid was estimated using $V = (4/3)\ \pi\ R^3$. Experiments were repeated 3 times with duplication in each experiment.

Western blotting analysis

The procedures for preparation of whole cell lysates and western blotting analysis have been previously described[23]. Mouse anti-human β-catenin, N-cadherin, vimentin, E-cadherin, ZO-1, CD133, CD44 and β-actin antibodies were used as primary antibodies. Signals were visualized using chemiluminescent substrate (ECL; Amersham, Arlington Heights, IL, United States). β-actin was used as an internal control. Images were scanned, followed by densitometry analysis with UN-SCAN-IT software (Silk Scientific Inc., Orem, UT, United States).

MTT assay

$CD133^+$ sphere-forming cells (SFCs) or parental MHCC97 cells were seeded in a 96-well plate pre-coated with 0.6% agarose at a density of 5000 cells/well as described previously[24]. One day after plating, various concentrations of BrMC (0.1, 0.3 1.0, 3.0 or 10.0 μmol/L) were added to each well and the culture continued for 48 h. After removal of the medium, cells were incubated with 5 mg/mL of MTT for 4 h. Cells were then extracted with acidic isopropanol and the absorbance at 570 nm (A_{570}) was measured by means of an enzyme-labeling instrument (EXL-800 type). The relative cell proliferation inhibition rate = (average A_{570} of the experimental group/average A_{570} of the control group) × 100%.

Matrigel invasion assay

The invasion ability of tumor cells was examined *in vitro* using a transwell chamber system with 8.0 μm pore polycarbonate filter inserts (Corning Coster, Cambridge, MA, United States). The lower side of the filter was coated with 10 μL gelatin (1 mg/mL), and the upper side was coated with 10 μL of Matrigel. Parental MHCC97 cells or LCSCs (2×10^3) were placed in the upper part of the filter. 10% fetal bovine serum was added in the lower part of the chamber as a chemical attractant. The chamber was then incubated at 37 ℃ for 48 h. Cells that could not invade through the filter were removed with a cotton swab. The cells in the lower part of the chamber were fixed with methanol and stained with crystal violet. The invasiveness of tumor cells was determined by counting the total number of cells on the lower side of the filter at 100 × magnification. In the drug-intervention experiment, cells were pretreated with different concentrations of BrMC for 24 h prior to the transwell chamber assay.

In vivo tumorigenicity experiments

Pathogen-free Balb/c-nu mice aged 5-6 wk were purchased from Shanghai Laboratory Animal Center (Shanghai, China). All animal studies were performed in accordance with the standard protocols approved by the Ethical Committee of Hunan Normal University and the Committee of Experimental Animal Feeding and Management. Mice were randomly divided into 3 groups (4 mice/group) and maintained under standard conditions, according to the standard protocols. Cells were suspended in serum free-DMEM/Matrigel (BD Biosciences, San Jose, CA, United States) mixture (1:1 volume). Each recipient Balb/c-nu mouse was inoculated subcutaneously with various numbers of $CD133^+$ SFCs (2×10^3, 1×10^4 and 1×10^5 cells) in one flank and parental MHCC97 cells (1×10^4, 1×10^5 and 1×10^6) in the other. Tumorigenicity experiments were terminated 2 mo after cell inoculation. Tumor size were measured with a caliper, and the volume was calculated using $V\ (mm^3) = L \times W^2 \times 0.5$. Harvested tumors were imaged and weighed immediately. Specimens from tumor tissue samples were fixed in 10% neutral buffered formalin, processed in paraffin blocks, and sectioned. The sections were stained with hematoxylin and eosin (HE) and examined for the histopathology.

For BrMC treatment studies, 5×10^4 LCSCs per mouse were injected subcutaneously. Two weeks after inoculation, animals were randomly divided into 4 groups. One group underwent daily gastric lavage with refined olive oil as control, and the other 3 groups were treated with 12.5, 25 or 50 mg/kg BrMC. After 20 d of treatment, living cells from the primary tumors were dissociated and injected into 3 groups of mice (4 mice per group). Each mouse was implanted with 5×10^4 cells from the control group and from the 50 mg/kg BrMC treated group in each flank. The growth of tumors was monitored, and tumor volumes were measured every 3 d. Animals were humanely sacrificed when the larger of the two tumors reached 500 mm^3.

Immunohistochemical examination

For immunohistochemical analysis of CD44 and CD133, tissues of the LCSCs derived-tumors in the nude mouse xenograft model were performed with formalin-fixed, paraffin-embedded sectioning as previously described by Moinfar *et al*[25]. After incubation with 1% non-fat dry milk in PBS (pH 7.4), the sections were then reacted with mouse anti-CD44 monoclonal antibody (1:250, Cell Signaling Technology Inc.) or mouse anti-CD133 monoclonal antibody (1:200, Abzoom, Dallas, TX, United States) for 1 h at room temperature followed by incubation with the secondary biotinylated antibody for 30 min. After washing, sections were subsequently incubated with streptavidin-peroxidase for 30 min. Finally, the results were vi-

sualized after a 15-min incubation with diaminobenzidine.

RNA interference

A control non-specific small interfering RNA (siRNA) (5'-GACTTCATAAGGCGCATGC-3') and β-catenin siRNA (5'-AGCUGAUAUUGAUGGACAGTT-3') were synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). Transfection of siRNA was carried out with Lipofectamine 2000 (Invitrogen Life Technologies) according to the procedure recommended by the manufacturer. Twenty-four hours after transfection, the cells were treated with DMSO (control) or BrMC at the indicated concentrations for 24 h. The cells were then collected and processed for western blotting and the tumorsphere formation assay.

RESULTS

Isolation and characterization of LCSCs derived from MHCC97 cell line

CD133 has previously been classified as a CSC marker in liver cancer. Therefore, we first isolated the $CD133^+$ subpopulation from MHCC97 cells by MACS. Following sorting, we examined the expression of CD133 by flow cytometry. As shown in Figure 1A, the sorted $CD133^+$ cells showed a high purity of 57.29% ± 4.61%, as compared with a purity of 1.02% ± 0.65% for $CD133^-$ counterparts and 7.21% ± 1.34% for non-sorted MHCC97 cells. To establish long-term cultures enriched in stem cells from sorted $CD133^+$, we performed the tumorsphere assay by culturing the cells in stem cell-conditioned medium. Within 6 d of culture, we obtained liver cancer spheroids both in $CD133^+$ cells and parental MHCC97 cells (Figure 1B). As shown in Table 1, the $CD133^+$ subpopulation exhibited a 2.7- and 2.5-fold enhancement in tumorsphere formation amount and size, respectively, compared with that of parental cells, whereas, $CD133^-$ counterparts could not grow as spheroids in the nonadherent and serum-free conditions.

To further confirm the stem cell properties and functions of the $CD133^+$ SFCs, we evaluated their self-renewal capacity and tumorigenic potential. First, we measured the capacity of single cells obtained from these $CD133^+$ dissociated spheres to form secondary tumorspheres. Within 9 d of culture, we obtained new LCSC spheroids of growing undifferentiated $CD133^+$ cells (Figure 1C). These suggest an *in vitro* self-renewing capacity of $CD133^+$ SFCs. In addition, $CD133^+$ SFCs also expressed an enhanced level of stem cell markers, CD133 and CD44, compared with their parental cells (Figure 1D). Next, we evaluated the tumorigenic potential of $CD133^+$ SFCs. We investigated the ability of $CD133^+$ SFCs and parental cells to give rise to tumors in Balb/c-nu mice. As many as 1×10^6 parental cells were needed to initiate stable tumor formation 39 d after injection, while, in contrast, as few as 1×10^4 $CD133^+$ SFCs were sufficient to generate visible tumors only 23 d post-injection (Table 2). These data indicate that $CD133^+$ SFCs, namely LCSCs, are more tumorigenic than their parental cells *in vivo*. Additionally, HE staining was performed and revealed similar histological characteristics in tumor xenografts derived from $CD133^+$ SFCs and their parental cells (Figure 1E).

BrMC inhibits proliferation and self-renewal of LCSCs derived from MHCC97 cell line

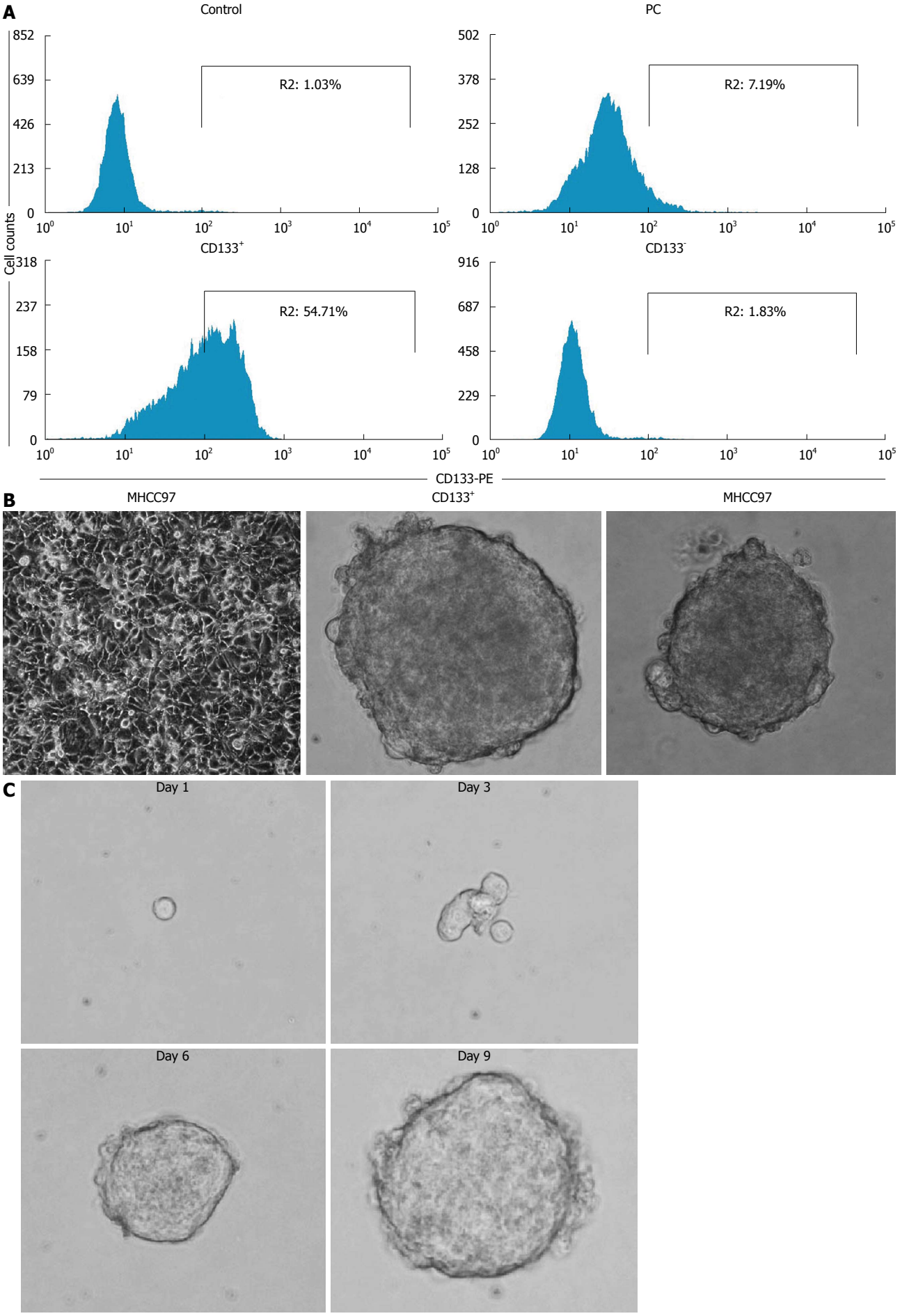
CSCs possess the property of limitless proliferative potential. A number of previous studies have demonstrated that some naturally-occurring polyphenol compounds such as genistein preferentially inhibit proliferation of pancreatic cancer stem cells[26]. In this study, we thus evaluated the anti-proliferative effects of BrMC on LCSCs derived from MHCC97 cell line by MTT assay. As shown in Figure 2A, when cells were treated with different concentrations of BrMC for 48 h, BrMC preferentially inhibited proliferation of LCSCs in a dose-dependent manner, with the IC_{50} around 0.5 μmol/L for LCSCs and 17.9 μmol/L for parental MHCC97 cells.

In order to evaluate whether BrMC could suppress the self-renewal of LCSCs derived from the MHCC97 cell line *in vitro*, we treated the primary tumorspheres with varying concentrations of BrMC and then removed the drug and cultured them for another passage to form the secondary spheres. Results showed that BrMC treatment resulted in a decrease both in tumorsphere number and size of LCSCs. Furthermore, a significant decrease in the number and size of the secondary tumorspheres indicated a reduced self-renewal capacity of these LCSCs by BrMC treatment (Figure 2B and C).

BrMC inhibits Epithelial-mesenchymal transition and invasion of LCSCs derived from MHCC97 cell line

Epithelial-mesenchymal transition (EMT) is an important process during metastasis of LCSCs. Therefore, we sought to examine whether morphological changes existed between LCSCs and parental MHCC97 cells cultured adherently *in vitro*. As observed in Figure 3A, LCSCs exhibited a spindle-like shape, while parental MHCC97 cells displayed a cobble-stone-like phenotype. However, treatment with 0.1 μmol/L BrMC suppressed EMT in LCSCs as morphological changes from a spindle-like shape to a cobble-stone-like appearance were displayed. Moreover, similar results were further confirmed by western blotting using specific antibodies against EMT-relative markers. Figure 3B shows that LCSCs expressed higher vimentin and N-cadherin protein levels, which are typically associated with mesenchymal cells, and lower expression of epithelium-associated E-cadherin and ZO-1 proteins. However, BrMC induced the upregulation of epithelial markers E-cadherin and ZO-1 and the downregulation of mesenchymal markers N-cadherin and vimentin after treatment for 24 h of LCSCs derived from MHCC97 cell line.

Since EMT has been identified as being associated with increased cancer cell invasion, we next evaluated the effect of BrMC on cell invasion of LCSCs *in vitro* using a transwell chamber coated with a Matrigel barrier. As


A
Control
PC
R2: 1.03%
R2: 7.19%
Cell counts
CD133+
CD133-
R2: 54.71%
R2: 1.83%
CD133-PE
B
MHCC97
CD133+
MHCC97
C
Day 1
Day 3
Day 6
Day 9

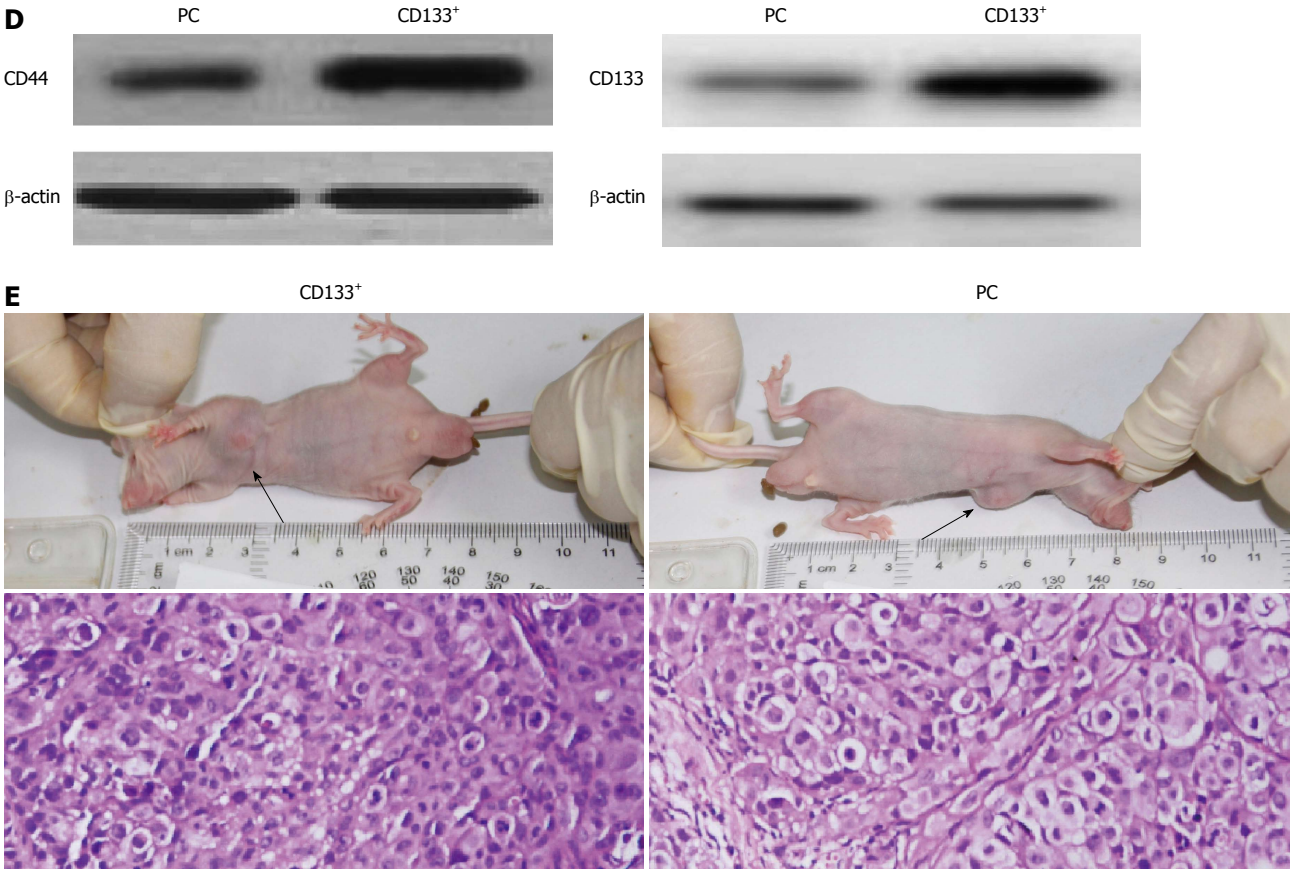


Figure 1 Isolation and characterization of liver cancer stem cells derived from the MHCC97 cell line. A: Flow cytometry analysis of CD133 expression following sorting. $CD133^+$ cells from MHCC97 cells formed liver cancer spheroids in stem cell-conditioned medium (200 × magnification); B: Anchorage-dependent growth of MHCC97 cells, tumor spheroid formed by $CD133^+$ cells, tumor spheroid formed by parental MHCC97 cells; C: Secondary tumorspheres formed by single cells from dissociated primary liver spheroids (400 × magnification); D: Expression of stem cell surface markers CD44 and CD133 in $CD133^+$ sphere-forming cells (SFCs) and parental cells; E: Hematoxylin-eosin staining revealed similar histological characteristics in tumor xenografts derived from $CD133^+$ SFCs and their parental cells (100 × magnification).

Table 1 Tumorsphere formation ability of $CD133^+$ cells derived from the MHCC97 cell line (mean ± SD, n = 3)

Cell line	Spheroid number/2 × 10^3 cells		Volume of spheroid (μm^3)	
	Parental cells	$CD133^+$ cells	Parental cells	$CD133^+$ cells
MHCC97	61 ± 26	167 ± 31[a]	153 ± 31	397 ± 45[a]

[a]P <0.05 *vs* parental MHCC97 cells.

shown in Figure 3C and 3D, BrMC significantly reduced the invasiveness capacity of LCSCs in a dose-dependent manner. These results demonstrated that BrMC possesses inhibitory effects on EMT and invasion in LCSCs derived from MHCC97 cell line.

BrMC eliminates LCSCs derived from MHCC97 cell line in vivo

In order to evaluate whether BrMC could target LCSCs *in vivo*, we utilized the xenograft model of LCSCs from MHCC 97 cells in Balb/c-nu mice. Two weeks after cell inoculation with 5×10^4 LCSCs resuspended in Matrigel, animals underwent daily gastric lavage with various concentrations of BrMC. After 20 d of treatment, tumors in 25 and 50 mg/kg BrMC-treated mice were less than 50% of the size of those in refined olive oil control animals (Figure 4A and B). Immunohistochemical analysis of CD44 and CD133 in LCSC-derived tumors revealed that the LCSC markers CD44 and CD133 were mainly expressed on the cell surface of the cancer cells, and that the tumors derived from $CD133^+$ SFCs showed significantly higher CD44 and CD133 positive rates than that of tumors derived from parental cells (Figure 4C). Furthermore, BrMC treatment can significantly decrease the CD44 and CD133 expression frequency of the tumors derived from LCSCs (Figure 4D).

To further confirm the results, we investigated the growth of secondary tumors in Balb/c-nu mice inoculated with tumor cells dissociated from primary tumor xenografts. In order to avoid possible variations due to heterogeneity, each recipient mouse was inoculated with 5×10^4 cells obtained from 50 mg/kg BrMC-treated tumors and another 5×10^4 cells obtained from control tumors in two opposite sides. Interestingly, we found that tumor cells from control animals exhibited rapid tumor re-growth, reaching a final tumor volume of 567-686 mm^3. However, the tumor cells from 50 mg/kg BrMC-

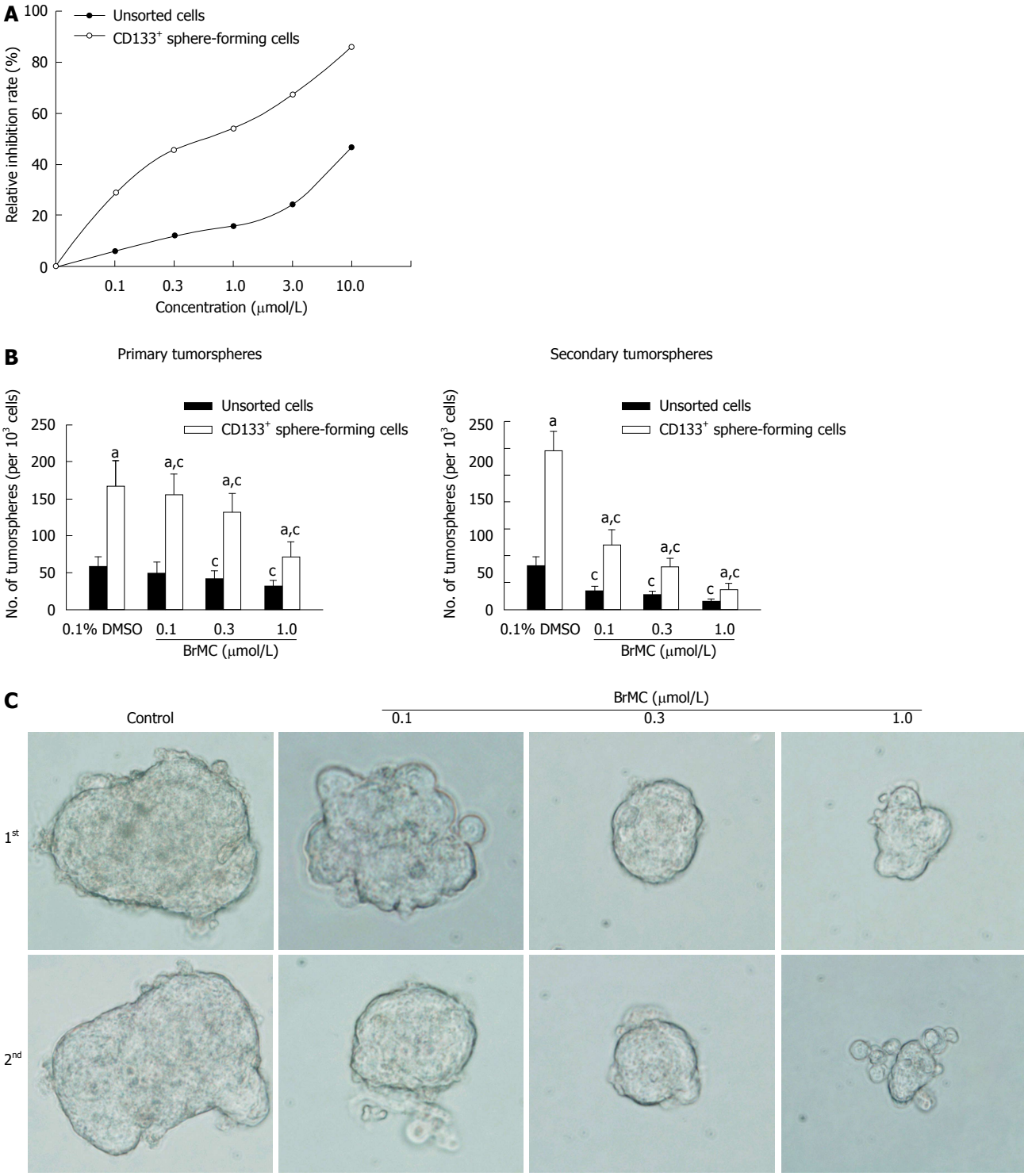
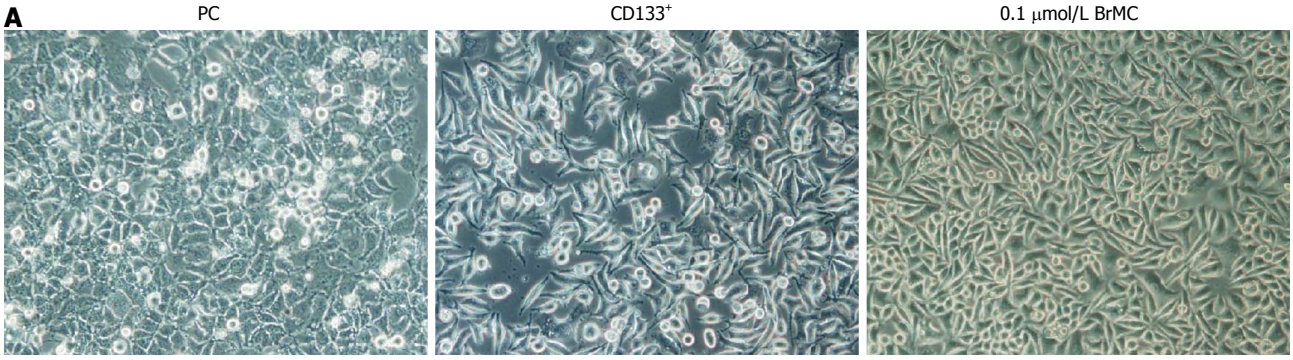


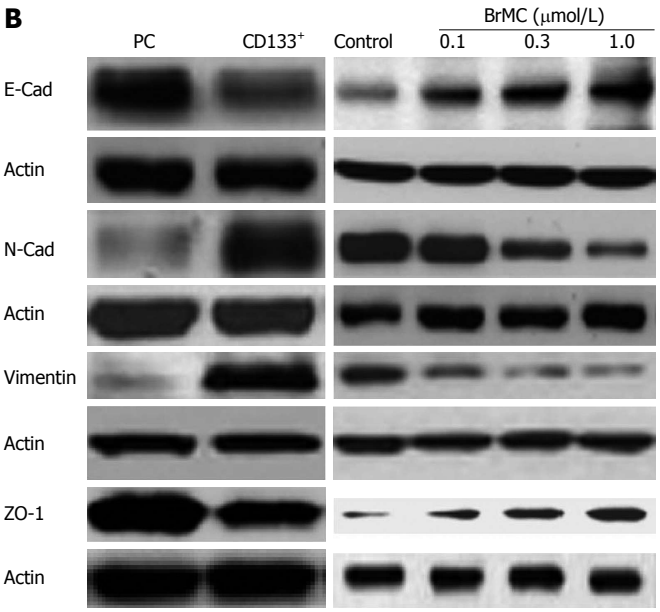
Figure 2 Effects of 8-bromo-7-methoxychrysin on cell proliferation and self-renewal. 8-bromo-7-methoxychrysin (BrMC) inhibited proliferation (A), self-renewal (B and C) of liver cancer stem cells derived from MHCC97 cell line (mean ± SD, $n = 3$). $^{a}P < 0.05$ *vs* unsorted MHCC97 cells treated with corresponding concentrations of BrMC. $^{c}P < 0.05$ *vs* corresponding 0.1% DMSO treated group. Tumor sphere morphology is shown as the phase contrast image (400 × magnification).

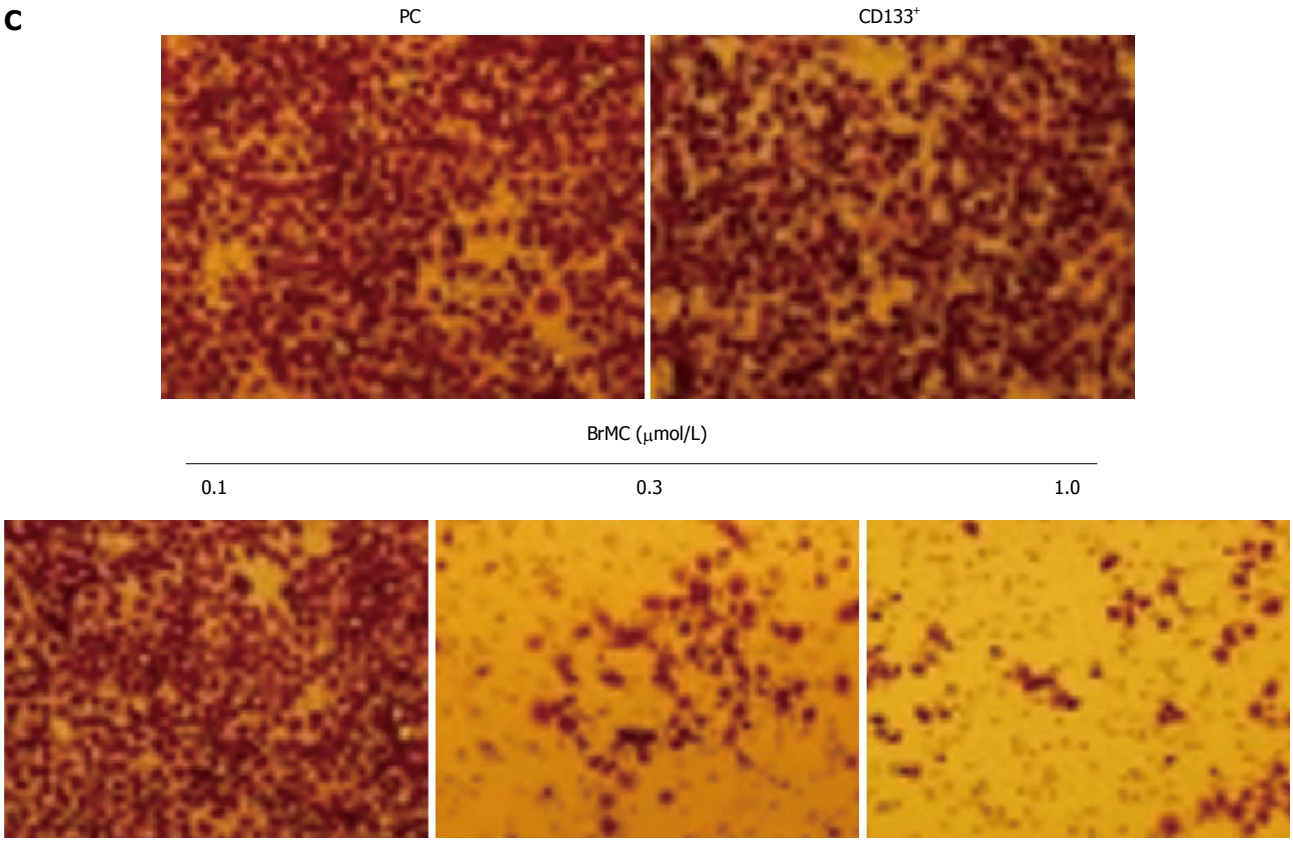
treated mice mostly failed to generate any tumors up to 33 d after inoculation (Table 3). These results suggest that BrMC was able to eliminate LCSCs in primary tumor xenografts, thereby inhibiting tumor regrowth in secondary inoculated mice.

BrMC inhibits self-renewal in LCSCs through modulation of β-catenin expression

To examine whether BrMC could regulate expression of stem cell markers of LCSCs, we determined the expression of CD44 and CD133 following BrMC treatment


A
PC
CD133+
0.1 μmol/L BrMC


B
BrMC (μmol/L)
PC
CD133+
Control
0.1
0.3
1.0
E-Cad
Actin
N-Cad
Actin
Vimentin
Actin
ZO-1
Actin


C
PC
CD133+
BrMC (μmol/L)
0.1
0.3
1.0

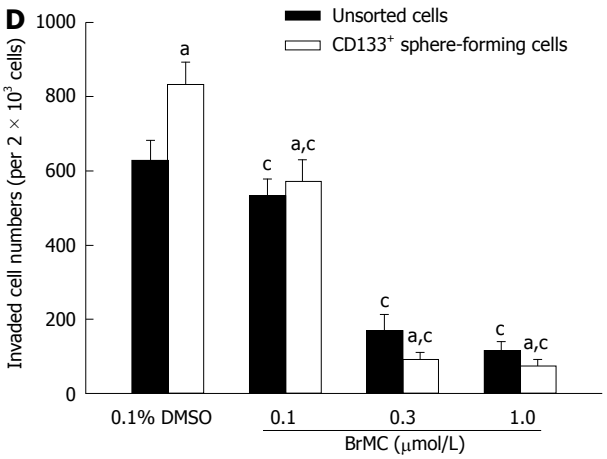


Figure 3 8-bromo-7-methoxychrysin inhibition of liver cancer stem cells. 8-bromo-7-methoxychrysin (BrMC) inhibited epithelial-mesenchymal transition (EMT, A and B) and invasion (C and D) of liver cancer stem cells derived from the MHCC97 cell line (mean ± SD, $n = 3$). Cell morphological changes associated with EMT are shown as the phase contrast image (200 × magnification). $^{a}P < 0.05$ *vs* unsorted MHCC97 cells treated with corresponding concentrations of BrMC, $^{c}P < 0.05$ *vs* corresponding 0.1% DMSO treated group.

by western blotting analysis. Results showed that BrMC downregulated CD44 and CD133 expression in a dose-dependent manner (Figure 5A). This was in accordance with our previous immunohistochemical analysis in LCSC-derived tumors (Figure 4D).

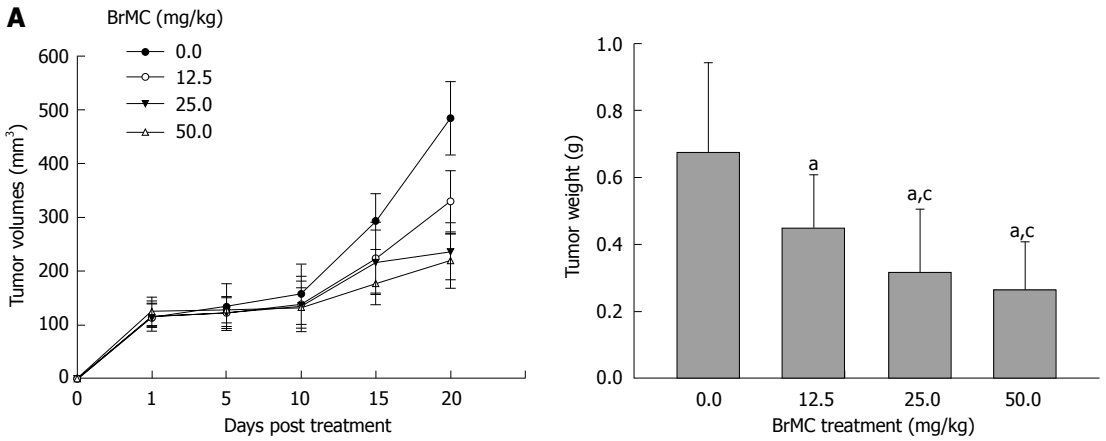
CD44 has been shown to be a downstream target of the β-catenin signaling pathway[27]. Wnt/β-catenin signaling has been implicated in the maintenance of CSCs of liver cancer[28]. Therefore, we measured the expression level of stem cell signal molecule β-catenin in LCSCs and parental MHCC97 cells, and examined whether β-catenin was downregulated by BrMC in LCSCs. Western blotting analysis showed that β-catenin was highly expressed in LCSCs compared with that of parental MHCC97 cells. We also found that BrMC (0.1, 0.3, 1.0 μmol/L) treatment resulted in a significant decrease in β-catenin expression of LCSCs (Figure 5B).

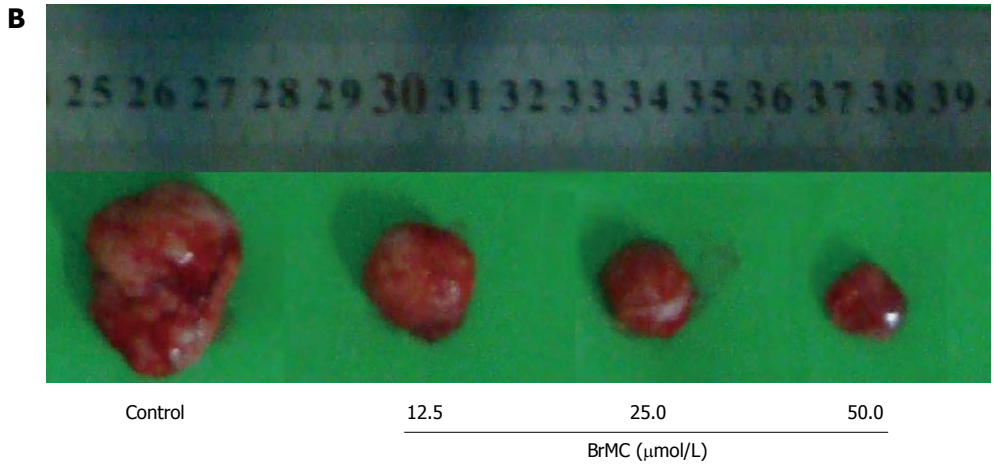
We further determined the role of β-catenin in the maintenance of self-renewal of LCSCs. Silencing of β-catenin by siRNA transfection resulted in less expression of β-catenin protein, as confirmed by Western blotting (Figure 5C). We also found that the downregulation of β-catenin expression significantly decreased the tumorsphere formation ability and inhibited expression of stem cell markers of LCSCs (Figure 5D and 5E). BrMC (0.1 μmol/L) plus β-catenin siRNA inhibited β-catenin expression to a greater degree compared to either alone (Figure 6A). Moreover, β-catenin siRNA potentiated the BrMC-induced decrease in tumorsphere formation of LCSCs (Figure 6B). We also treated LCSCs with Wnt3a, a ligand known to activate the Wnt/β-catenin pathway. As expected, Wnt3a induced β-catenin stabilization and resulted in a corresponding up-regulation of β-catenin in LCSCs (Figure 6C). This upregulation of β-catenin attenuated BrMC-induced downregulation of β-catenin and stem cell markers and antagonized BrMC-induced inhibition of self-renewal of LCSCs (Figure 6D, 6E and 6F). Taken together, these results provide some molecular evidence suggesting that the downregulation of β-catenin expression may contribute to the inhibitory effects of BrMC on LCSCs.

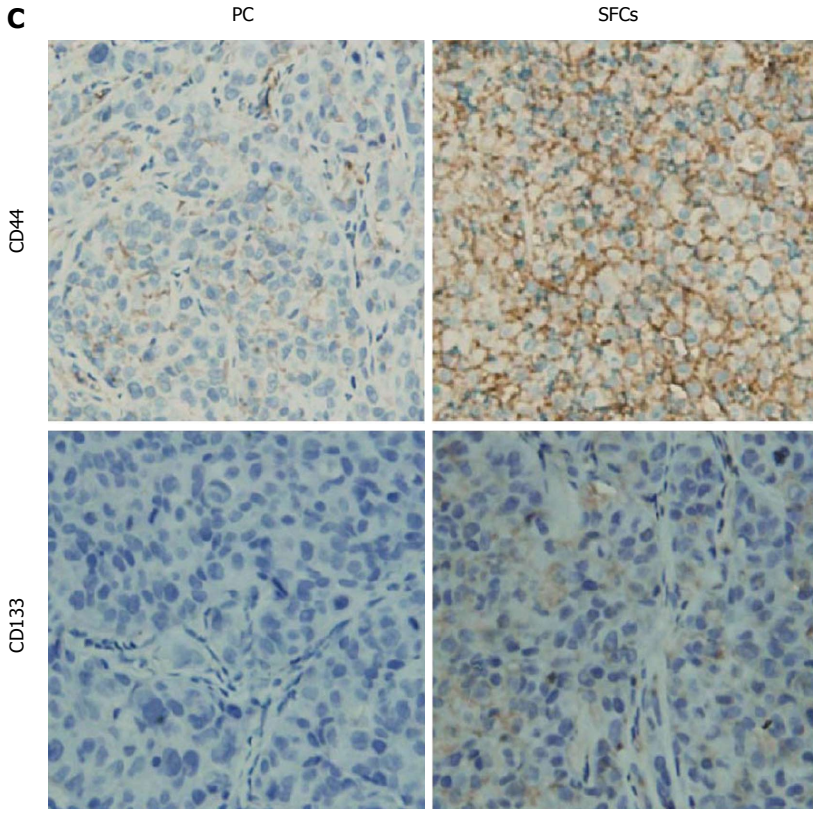
DISCUSSION

Cancer stem cells are defined as a minor population of tumorigenic cells that are capable of continuous self-renewal and differentiation, and undergo unlimited proliferation, giving rise to new tumors[29,30]. Therefore, finding compound(s) that are capable of inhibiting or killing the CSCs is extremely important to overcome tumor resistance, reduce relapse, and eventually improve overall survival. Our previous study has shown that BrMC possessed promising inhibitory effects on proliferation and apoptosis of colon, gastric and liver cancer cells. In the current study, we first successfully isolated and identified LCSCs from the liver cancer MHCC97 cell line. Further, we showed for the first time that BrMC could preferentially inhibit proliferation and self-renewal, and suppress EMT and invasion of LCSCs. Moreover, BrMC was able to eradicate LCSCs *in vivo*, as assessed by an *in vivo* tumorigenicity assay using primary and secondary Balb/c-nu mouse models. Secondly, we found that the inhibitory effects of BrMC on stem cell function and properties of LCSCs were mediated by inhibition of β-catenin pathways: β-catenin siRNA transfection and BrMC were synergistic in inhibiting the self-renewal of LCSCs. Conversely, the inhibition of Wnt3a in LCSCs resulted in an opposite effect.

More recently, CD133 has been used as a surface marker of CSCs in various solid tumors, including liver cancer. However, the function of CD133 is not entirely known yet. Thus, the single phenotypic marker CD133 is not sufficient to identify LCSCs. Tumorsphere culture may provide an alternative approach to identify and enrich LCSCs. Under non-adherent serum-free conditions *in vitro*, most tumor cells undergo programmed cell death,


A
BrMC (mg/kg)
0.0
12.5
25.0
50.0
Tumor volumes (mm3)
600
500
400
300
200
100
0
0
1
5
10
15
20
Days post treatment
Tumor weight (g)
1.0
0.8
0.6
0.4
0.2
0.0
a
a,c
a,c
0.0
12.5
25.0
50.0
BrMC treatment (mg/kg)


B
Control
12.5
25.0
50.0
BrMC (μmol/L)


C
PC
SFCs
CD44
CD133

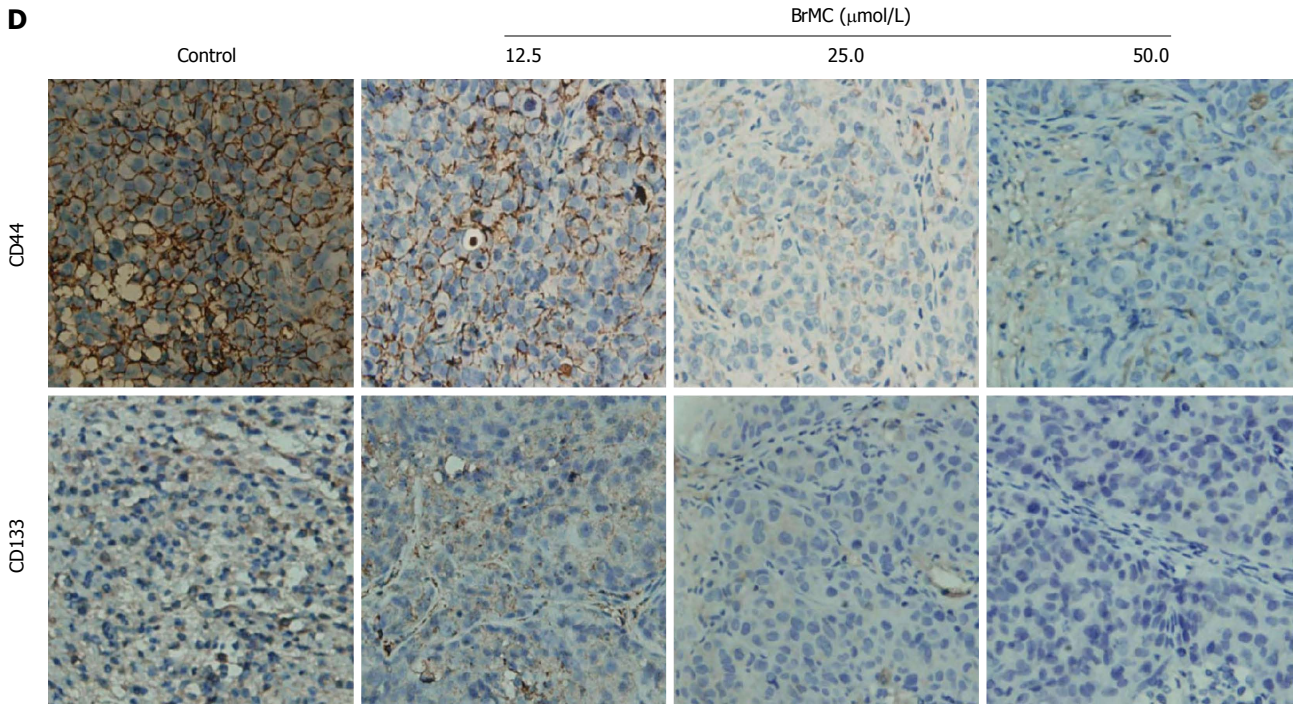


Figure 4 8-bromo-7-methoxychrysin eliminated liver cancer stem cells derived from MHCC97 cell line *in vivo*. Effects of 8-bromo-7-methoxychrysin (BrMC) on growth of primary and secondary tumor xenografts derived from liver cancer stem cells (LCSCs) (A and B, mean ± SD, $n = 12$). $^{a}P < 0.05$ *vs* refined olive oil treatment model, $^{c}P < 0.05$ *vs* treatment with 12.5 mg/kg BrMC. Immunohistochemical analysis of CD44 and CD133 in LCSC-derived tumors before and after BrMC treatment (C and D).

Table 2 Tumorigenicity of CD133$^+$ sphere forming cell derived from MHCC97 cells in Balb/c-nu mice

Cell type	No. inoculated cells	Tumor incidence[1]	Latency (d)[2]
Parental cells	1×10^4	0/4	-
	1×10^5	0/4	-
	1×10^6	4/4	39
CD133$^+$ SFCs	2×10^3	3/4	31
	1×10^4	4/4	23
	1×10^5	4/4	8

[1]Number of tumors detected/number of injections; [2]Approximate number of days from tumor cell injection to appearance of a tumor. SFC: Sphere forming cells.

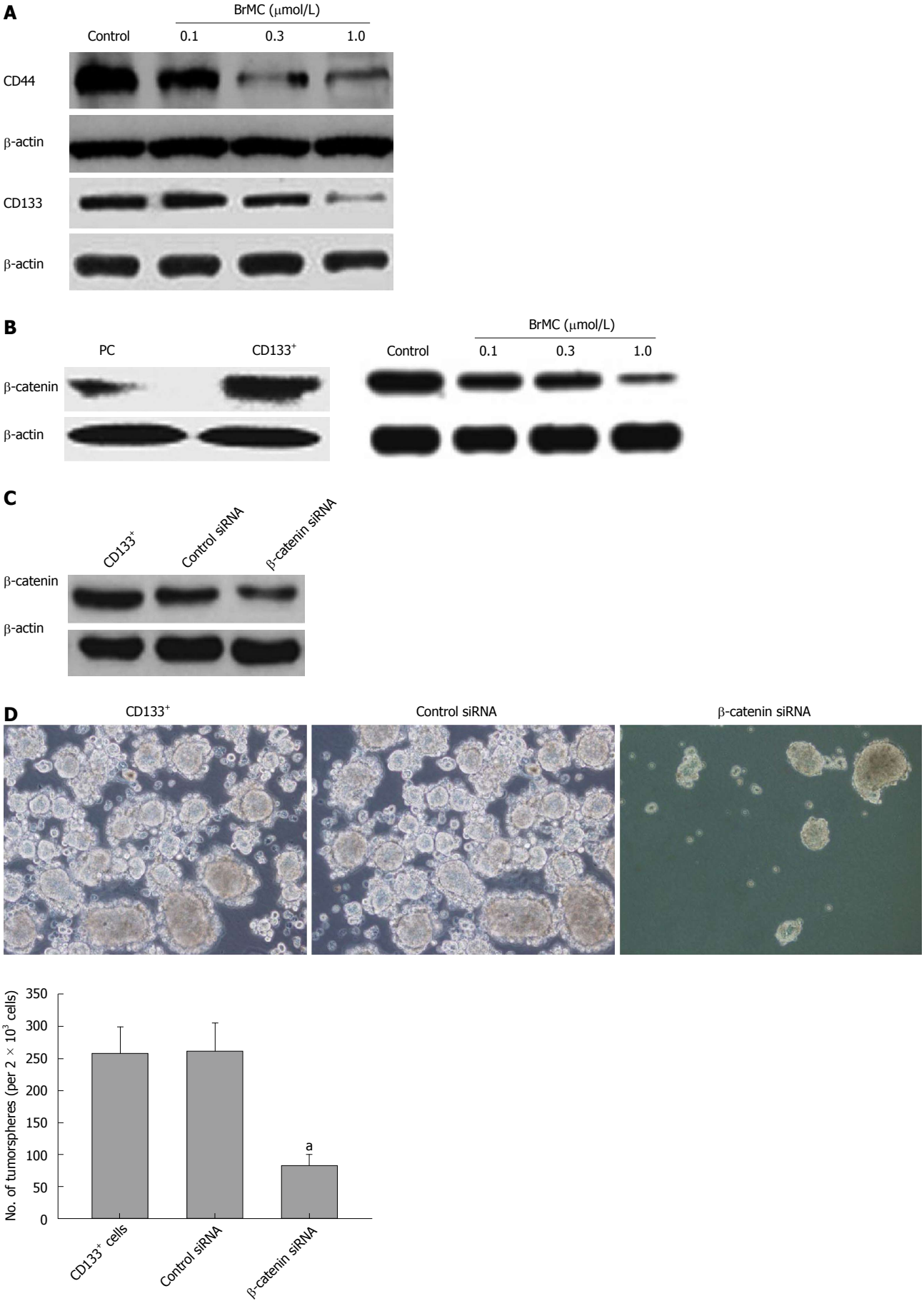
Table 3 Effects of 8-bromo-7-methoxychrysin on growth of secondary tumors in Balb/c-nu mice inoculated with tumor cells obtained from primary xenografts

Time (d)	Tumor incidence[1]		Volume (mm^3)	
	Control	BrMC-treated group	Control	BrMC-treated group
1	0/12	0/12	-	-
3	0/12	0/12	-	-
6	4/12	0/12	28 ± 12	-
12	9/12	0/12	134 ± 29	-
15	12/12	3/12	321 ± 63	16 ± 8
18	12/12	3/12	532 ± 96	33 ± 16
21	8/8	2/8	351 ± 67	23 ± 13
24	8/8	2/8	593 ± 131	32 ± 24
27	4/4	1/4	264 ± 91	54
30	4/4	1/4	387 ± 114	67
33	4/4	1/4	567 ± 126	82

[1]Number of tumors detected/number of injections. BrMC: 8-bromo-7-methoxychrysin

whereas the rare CSCs divide to generate multicellular 3-dimensional spheres[31,32]. This assay is a powerful tool to enrich CSCs and further assess the functional properties of the isolated CSCs. By employing a combination of this technique and MACS based on the CD133$^+$ surface marker, we have successfully obtained the putative LCSCs, namely CD133$^+$ SFCs, from the MHCC97 cell line. We demonstrated that these CD133$^+$ SFCs possess stem-like properties, including self-renewal, initiation of tumor growth in mice at very low cell numbers and a higher expression level of stem cell marker compared with their parental cells. These data indicated that the method which we used to isolate and indentify LCSCs from liver cancer cell lines may be faster, more economic and effective, compared with methods based on two or more surface markers.

The Wnt/β-catenin pathway is one of the key pathways that modulates stem cell self-renewal[14]. For example, overexpression of β-catenin enhanced self-renewal preferentially and mediated radiation resistance of Sca1$^+$ progenitors in an immortalized mammary gland cell line[33]. Hallett *et al*[34] reported that pharmacological inhibitors of Wnt/β-catenin signaling could inhibit the viability and or self-renewal of breast tumor-initiating cells, and target breast tumor-initiating cells in a Her2/Neu mouse model of breast cancer. Consistent with these previous reports, we found that downregulation of β-catenin


A
BrMC (μmol/L)
Control
0.1
0.3
1.0
CD44
β-actin
CD133
β-actin
B
PC
CD133+
BrMC (μmol/L)
Control
0.1
0.3
1.0
β-catenin
β-actin
C
CD133+
Control siRNA
β-catenin siRNA
β-catenin
β-actin
D
CD133+
Control siRNA
β-catenin siRNA
No. of tumorspheres (per 2 × 10³ cells)
350
300
250
200
150
100
50
0
a
CD133+ cells
Control siRNA
β-catenin siRNA

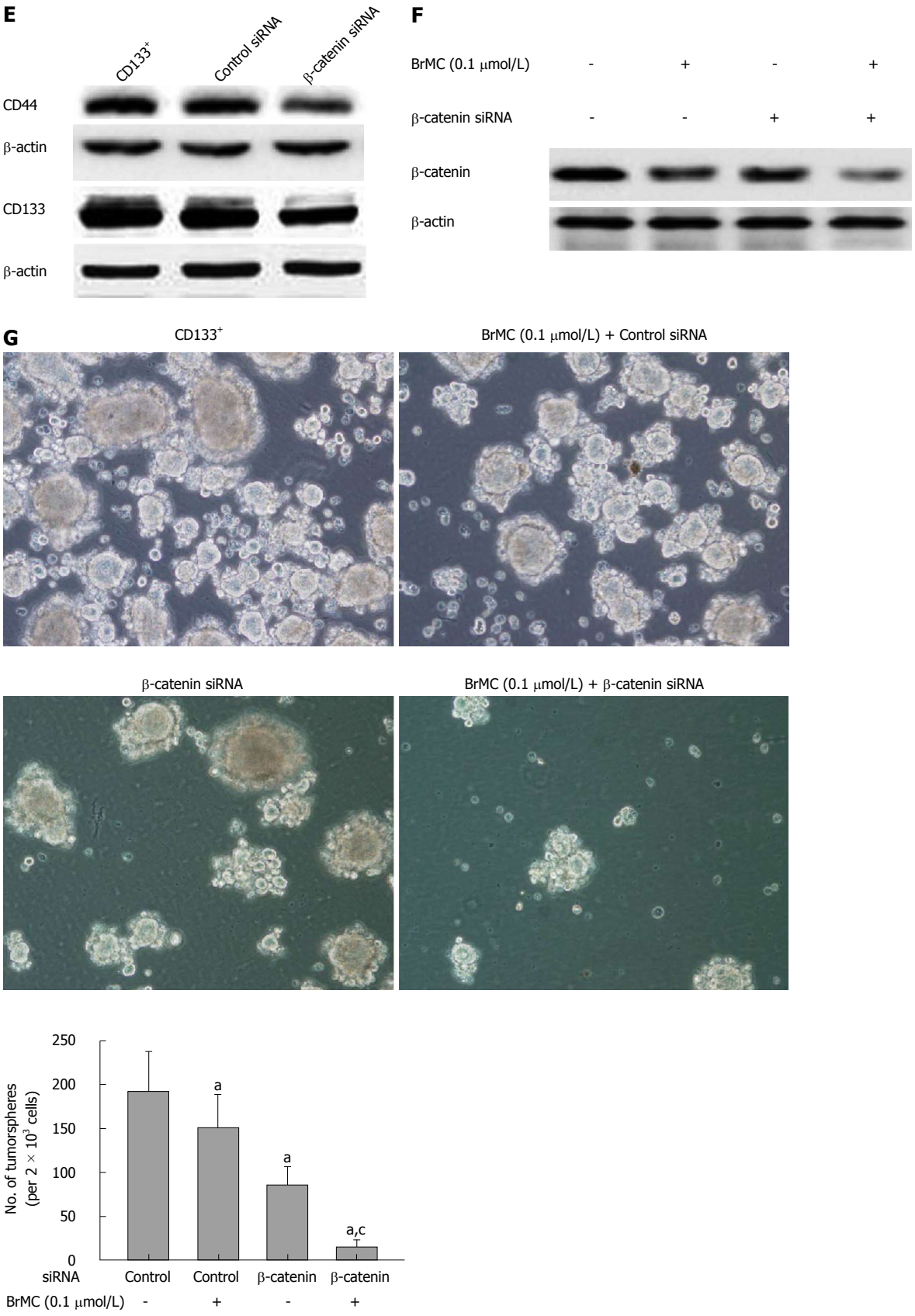


Figure 5 β-catenin siRNA synergized the inhibitory effects of 8-bromo-7-methoxychrysin. 8-bromo-7-methoxychrysin (BrMC) downregulated CD44 and CD133 expression in liver cancer stem cells in a concentration-dependent manner (A). β-catenin was highly expressed in $CD133^+$ sphere forming cells (SFCs) and was downregulated by BrMC treatment (B). β-catenin siRNA decreased the protein level of β-catenin (C) and stem cell markers (E), and significantly inhibited self-renewal capacity (D) of $CD133^+$ SFCs (mean ± SD, $n = 3$). $^aP < 0.05$ *vs* $CD133^+$ SFCs or control siRNA transfected $CD133^+$ SFCs. BrMC enhanced β-catenin siRNA induced downregulation of β-catenin expression (F) and inhibition of self-renewal capacity (G) in $CD133^+$ SFCs (mean ± SD, $n = 3$). $^aP < 0.05$ *vs* $CD133^+$ SFCs derived from the MHCC97 cell line. $^cP < 0.05$ *vs* 0.1 μmol/L BrMC or β-catenin siRNA treated $CD133^+$ SFCs.

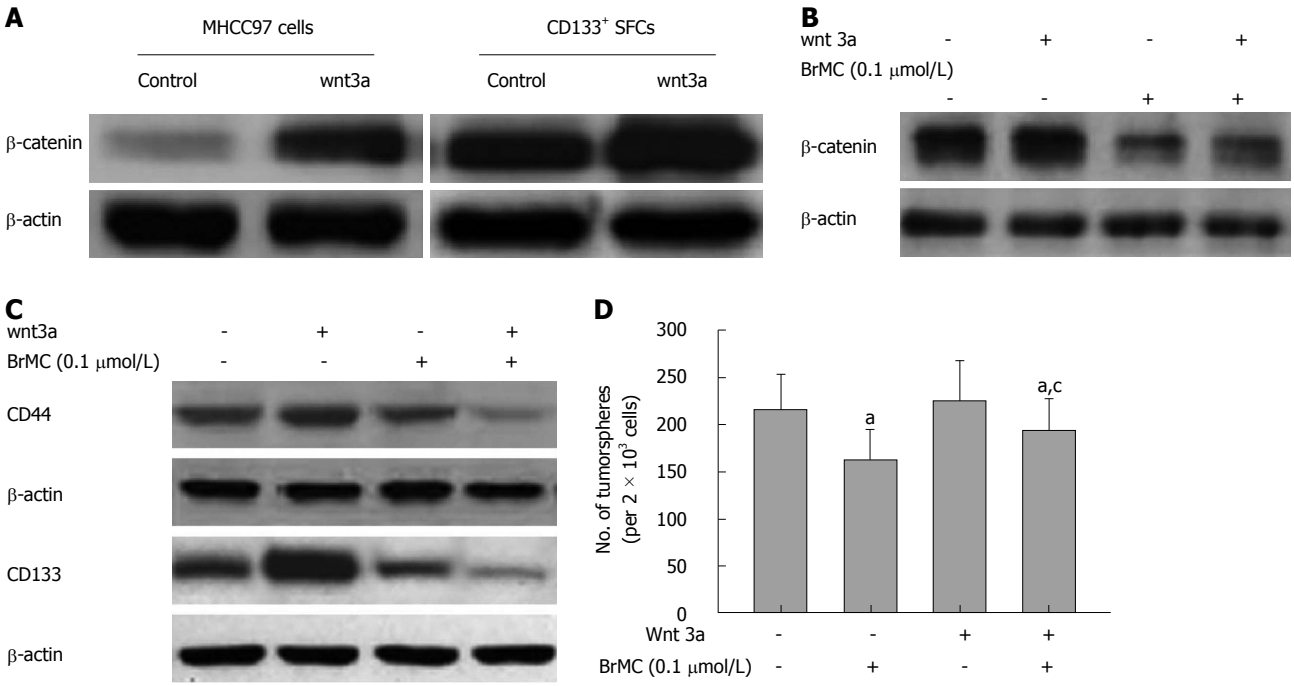


Figure 6 Wnt3a treatment antagonized the inhibitory effects of 8-bromo-7-methoxychrysin. Wnt3a treatment resulted in an increase in the expression of β-catenin in both liver cancer stem cells (LCSCs) and parental MHCC97 cells (A) and attenuated the effects of 8-bromo-7-methoxychrysin (BrMC) on the expression of β-catenin (B) and stem cell markers (C), and self-renewal capacity (D) of LCSCs derived from the MHCC97 cell line. $^{a}P < 0.05$ *vs* CD133^{+} SFCs, $^{c}P < 0.05$ *vs* 0.1 μmol/L BrMC or β-catenin siRNA alone treated group.

by BrMC resulted in inhibition of CSC function and characteristics of LCSCs, such as significant inhibition of proliferation and self-renewal, suppression of EMT and invasiveness, downregulation of the expression of stem cell markers of LCSCs, and further efficacious promotion of the elimination of LCSCs *in vivo*.

Previous studies have shown that activated Akt was able to phosphorylate Ser9 on GSK3β, which may decrease the activity of GSK3β, thereby leading to stabilization of β-catenin in the cytoplasm[35]. Chrysin was reported to induce apoptosis through caspase activation and Akt inactivation in leukemia cells[36]. Our previous study also demonstrated that 5,7-dihydroxy-8-nitrochrysin, another synthetic chrysin analogue, could induce activation and nuclear localization of FOXO3a, which was associated with reduced levels of Akt phosphorylation. Therefore, we speculate that the downregulation of β-catenin by BrMC probably occurs *via* reduced levels of Akt and activation of GSK3β, with the consequent degradation of β-catenin. Wnt3a treatment can induce stabilization of β-catenin, with entry into the nucleus and subsequent activation of the β-catenin pathway. Thus, Wnt3a treatment can antagonize the inhibitory effects of BrMC on self-renewal of LCSCs. On the other hand, Su *et al*[37] reported that genistein increases levels of membrane E-cadherin and E-cadherin-β-catenin cell adhesion complex, and eventually attenuates β-catenin signaling in mammary epithelial cells. We also found that E-cadherin, an epithelial marker, was upregulated by BrMC in LCSCs. E-cadherin is known to anchor and to sequester β-catenin in the membrane and prevent its activation. Therefore, we suppose that this inactivation of β-catenin by upregulation of E-cadherin can also contribute to the inhibitory effects of LCSCs by BrMC. Interestingly, the inhibition of β-catenin at the protein level was not optimal, as treatment of β-catenin siRNA can further downregulate β-catenin at the transcription level and synergize the inhibition of self-renewal of LCSCs induced by BrMC.

In conclusion, we have presented supportive evidence for the first time that BrMC, a novel synthetic chrysin analogue, can target LCSCs both *in vitro* and *in vivo*. Furthermore, our study identified the downregulation of β-catenin expression by BrMC as one of the possible mechanisms for its efficacy. These studies support the use of BrMC for liver cancer chemoprevention or chemotherapy. These findings provide a strong rationale for preclinical and subsequent clinical evaluation of BrMC for liver cancer therapy.

COMMENTS

Background

Liver cancer is the fifth most common cancer in the world and the third leading cause of cancer-related death. Recent studies indicated that cancer stem cells (CSCs) may be responsible for tumor recurrence and drug-resistance. Therefore, the identification of a compound that can target liver CSCs (LCSCs) is one of the main steps in improving overall survival of liver cancer patients.

Research frontiers

More recently, a number of studies have found that some dietary compounds can directly or indirectly affect CSC self-renewal pathways. 8-bromo-7-methoxychrysin (BrMC) is a synthetic derivative of chrysin, and their previous study have demonstrated the effect of BrMC on the inhibition of proliferation

and induction of apoptosis in colon, gastric and liver cancer cells was stronger than that of chrysin. However, the inhibitory effects of BrMC on the characteristics of CSCs have not been reported yet.

Innovations and breakthroughs

The authors firstly showed that BrMC, a novel synthetic chrysin analogue, was able to inhibit cancer stem cell-like properties of LCSCs and eliminate LCSCs *in vivo*. They also found that BrMC significantly decreased β-catenin expression in LCSCs and knockdown of β-catenin expression could synergize the inhibition of self-renewal of LCSCs induced by BrMC. The downregulation of β-catenin expression appears to contribute to the inhibitory effects of BrMC on the properties of LCSCs.

Applications

The present study provided strong evidences for the first time that BrMC was able to target LCSCs both *in vitro* and *in vivo*. These studies support the use of BrMC for liver cancer chemoprevention or chemotherapy.

Terminology

Chrysin (5,7-dihydroxyflavone), a naturally wide distributed flavonoid, has been reported to possess anti-cancer activities. BrMC is a novel synthetic chrysin analogue.

Peer review

This manuscript concludes that 8-bromo-7-methoxychrysin can inhibit the functions and characteristics of liver cancer stem cells derived from liver cancer MHCC97 cell line through downregulation of β-catenin expression. It is a good research with necessary information.

REFERENCES

1 **Dudeck O**, Ricke J. Advances in regional chemotherapy of the liver. *Expert Opin Drug Deliv* 2011; **8**: 1057-1069 [PMID: 21506902 DOI: 10.1517/17425247.2011.574125]

2 **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]

3 **Sanyal AJ**, Yoon SK, Lencioni R. The etiology of hepatocellular carcinoma and consequences for treatment. *Oncologist* 2010; **15** Suppl 4: 14-22 [PMID: 21115577 DOI: 10.1634/theoncologist.2010-S4-14]

4 **Villanueva A**, Llovet JM. Targeted therapies for hepatocellular carcinoma. *Gastroenterology* 2011; **140**: 1410-1426 [PMID: 21406195 DOI: 10.1053/j.gastro.2011.03.006]

5 **Oishi N**, Wang XW. Novel therapeutic strategies for targeting liver cancer stem cells. *Int J Biol Sci* 2011; **7**: 517-535 [PMID: 21552419]

6 **Shafee N**, Smith CR, Wei S, Kim Y, Mills GB, Hortobagyi GN, Stanbridge EJ, Lee EY. Cancer stem cells contribute to cisplatin resistance in Brca1/p53-mediated mouse mammary tumors. *Cancer Res* 2008; **68**: 3243-3250 [PMID: 18451150 DOI: 10.1158/0008-5472.CAN-07-5480]

7 **Hambardzumyan D**, Squatrito M, Holland EC. Radiation resistance and stem-like cells in brain tumors. *Cancer Cell* 2006; **10**: 454-456 [PMID: 17157785 DOI: 10.1016/j.ccr.2006.11.008]

8 **Suetsugu A**, Nagaki M, Aoki H, Motohashi T, Kunisada T, Moriwaki H. Characterization of CD133+ hepatocellular carcinoma cells as cancer stem/progenitor cells. *Biochem Biophys Res Commun* 2006; **351**: 820-824 [PMID: 17097610 DOI: 10.1016/j.bbrc.2006.10.128]

9 **Ma S**, Chan KW, Hu L, Lee TK, Wo JY, Ng IO, Zheng BJ, Guan XY. Identification and characterization of tumorigenic liver cancer stem/progenitor cells. *Gastroenterology* 2007; **132**: 2542-2556 [PMID: 17570225 DOI: 10.1053/j.gastro.2007.04.025]

10 **Yin S**, Li J, Hu C, Chen X, Yao M, Yan M, Jiang G, Ge C, Xie H, Wan D, Yang S, Zheng S, Gu J. CD133 positive hepatocellular carcinoma cells possess high capacity for tumorigenicity. *Int J Cancer* 2007; **120**: 1444-1450 [PMID: 17205516 DOI: 10.1002/ijc.22476]

11 **Zhu Z**, Hao X, Yan M, Yao M, Ge C, Gu J, Li J. Cancer stem/progenitor cells are highly enriched in CD133+CD44+ population in hepatocellular carcinoma. *Int J Cancer* 2010; **126**: 2067-2078 [PMID: 19711346 DOI: 10.1002/ijc.24868]

12 **Chen Y**, Yu D, Zhang H, He H, Zhang C, Zhao W, Shao RG. CD133(+)EpCAM(+) phenotype possesses more characteristics of tumor initiating cells in hepatocellular carcinoma Huh7 cells. *Int J Biol Sci* 2012; **8**: 992-1004 [PMID: 22904667 DOI: 10.7150/ijbs.4454ijbsv08p0992]

13 **Ma S**, Tang KH, Chan YP, Lee TK, Kwan PS, Castilho A, Ng I, Man K, Wong N, To KF, Zheng BJ, Lai PB, Lo CM, Chan KW, Guan XY. miR-130b Promotes CD133(+) liver tumor-initiating cell growth and self-renewal via tumor protein 53-induced nuclear protein 1. *Cell Stem Cell* 2010; **7**: 694-707 [PMID: 21112564 DOI: 10.1016/j.stem.2010.11.010]

14 **Li Y**, Wicha MS, Schwartz SJ, Sun D. Implications of cancer stem cell theory for cancer chemoprevention by natural dietary compounds. *J Nutr Biochem* 2011; **22**: 799-806 [PMID: 21295962 DOI: 10.1016/j.jnutbio.2010.11.001]

15 **Green JM**, Alvero AB, Kohen F, Mor G. 7-(O)-Carboxymethyl daidzein conjugated to N-t-Boc-hexylenediamine: a novel compound capable of inducing cell death in epithelial ovarian cancer stem cells. *Cancer Biol Ther* 2009; **8**: 1747-1753 [PMID: 19738422]

16 **Zhang L**, Li L, Jiao M, Wu D, Wu K, Li X, Zhu G, Yang L, Wang X, Hsieh JT, He D. Genistein inhibits the stemness properties of prostate cancer cells through targeting Hedgehog-Gli1 pathway. *Cancer Lett* 2012; **323**: 48-57 [PMID: 22484470 DOI: 10.1016/j.canlet.2012.03.037]

17 **Khoo BY**, Chua SL, Balaram P. Apoptotic effects of chrysin in human cancer cell lines. *Int J Mol Sci* 2010; **11**: 2188-2199 [PMID: 20559509 DOI: 10.3390/ijms11052188]

18 **Zheng X**, Meng WD, Xu YY, Cao JG, Qing FL. Synthesis and anticancer effect of chrysin derivatives. *Bioorg Med Chem Lett* 2003; **13**: 881-884 [PMID: 12617913]

19 **Xiang HL**, ZX, Cao JG. Induction of apoptosis of human gastric carcinoma SGC-790 cell line by 8-bromo-7-mehoxychrysin. *Zhongguo Yaolixue Tongbao* 2008; **24**: 1370-1372

20 **Ai XH**, Zheng X, Tang XQ, Sun L, Zhang YQ, Qin Y, Liu HQ, Xia H, Cao JG. Induction of apoptosis of human gastric carcinoma SGC-7901 cell line by 5, 7-dihydroxy-8-nitrochrysin in vitro. *World J Gastroenterol* 2007; **13**: 3824-3828 [PMID: 17657836]

21 **Yang XH**, Zheng X, Cao JG, Xiang HL, Liu F, Lv Y. 8-Bromo-7-methoxychrysin-induced apoptosis of hepatocellular carcinoma cells involves ROS and JNK. *World J Gastroenterol* 2010; **16**: 3385-3393 [PMID: 20632440]

22 **Willert K**, Brown JD, Danenberg E, Duncan AW, Weissman IL, Reya T, Yates JR, Nusse R. Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature* 2003; **423**: 448-452 [PMID: 12717451 DOI: 10.1038/nature01611]

23 **Ning Y**, Li Q, Xiang H, Liu F, Cao J. Apoptosis induced by 7-difluoromethoxyl-5,4'-di-n-octyl genistein via the inactivation of FoxM1 in ovarian cancer cells. *Oncol Rep* 2012; **27**: 1857-1864 [PMID: 22447287 DOI: 10.3892/or.2012.1739]

24 **Choi JN**, Kim D, Choi HK, Yoo KM, Kim J, Lee CH. 2'-hydroxylation of genistein enhanced antioxidant and antiproliferative activities in mcf-7 human breast cancer cells. *J Microbiol Biotechnol* 2009; **19**: 1348-1354 [PMID: 19996686]

25 **Moinfar F**, Okcu M, Tsybrovskyy O, Regitnig P, Lax SF, Weybora W, Ratschek M, Tavassoli FA, Denk H. Androgen receptors frequently are expressed in breast carcinomas: potential relevance to new therapeutic strategies. *Cancer* 2003; **98**: 703-711 [PMID: 12910513 DOI: 10.1002/cncr.11532]

26 **Bao B**, Wang Z, Ali S, Kong D, Banerjee S, Ahmad A, Li Y, Azmi AS, Miele L, Sarkar FH. Over-expression of FoxM1 leads to epithelial-mesenchymal transition and cancer stem cell phenotype in pancreatic cancer cells. *J Cell Biochem* 2011; **112**: 2296-2306 [PMID: 21503965 DOI: 10.1002/jcb.23150]

27 **Li J**, Zhou BP. Activation of β-catenin and Akt pathways by Twist are critical for the maintenance of EMT associated cancer stem cell-like characters. *BMC Cancer* 2011; **11**: 49 [PMID: 21284870 DOI: 10.1186/1471-2407-11-49]

28 **Yang W**, Yan HX, Chen L, Liu Q, He YQ, Yu LX, Zhang SH, Huang DD, Tang L, Kong XN, Chen C, Liu SQ, Wu MC, Wang HY. Wnt/beta-catenin signaling contributes to activation of normal and tumorigenic liver progenitor cells. *Cancer Res* 2008; **68**: 4287-4295 [PMID: 18519688 DOI: 10.1158/0008-5472.CAN-07-6691]

29 **Zhou BB**, Zhang H, Damelin M, Geles KG, Grindley JC, Dirks PB. Tumour-initiating cells: challenges and opportunities for anticancer drug discovery. *Nat Rev Drug Discov* 2009; **8**: 806-823 [PMID: 19794444 DOI: 10.1038/nrd2137]

30 **Curtin JC**, Lorenzi MV. Drug discovery approaches to target Wnt signaling in cancer stem cells. *Oncotarget* 2010; **1**: 552-566 [PMID: 21317452]

31 **Singh SK**, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman RM, Cusimano MD, Dirks PB. Identification of human brain tumour initiating cells. *Nature* 2004; **432**: 396-401 [PMID: 15549107 DOI: 10.1038/nature03128]

32 **Ponti D**, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D, Pilotti S, Pierotti MA, Daidone MG. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res* 2005; **65**: 5506-5511 [PMID: 15994920 DOI: 10.1158/0008-5472.CAN-05-0626]

33 **Chen MS**, Woodward WA, Behbod F, Peddibhotla S, Alfaro MP, Buchholz TA, Rosen JM. Wnt/beta-catenin mediates radiation resistance of Sca1+ progenitors in an immortalized mammary gland cell line. *J Cell Sci* 2007; **120**: 468-477 [PMID: 17227796 DOI: 10.1242/jcs.03348]

34 **Hallett RM**, Kondratyev MK, Giacomelli AO, Nixon AM, Girgis-Gabardo A, Ilieva D, Hassell JA. Small molecule antagonists of the Wnt/β-catenin signaling pathway target breast tumor-initiating cells in a Her2/Neu mouse model of breast cancer. *PLoS One* 2012; **7**: e33976 [PMID: 22470504 DOI: 10.1371/journal.pone.0033976PONE-D-11-22474]

35 **Cohen P**, Frame S. The renaissance of GSK3. *Nat Rev Mol Cell Biol* 2001; **2**: 769-776 [PMID: 11584304 DOI: 10.1038/35096075]

36 **Woo KJ**, Jeong YJ, Park JW, Kwon TK. Chrysin-induced apoptosis is mediated through caspase activation and Akt inactivation in U937 leukemia cells. *Biochem Biophys Res Commun* 2004; **325**: 1215-1222 [PMID: 15555556 DOI: 10.1016/j.bbrc.2004.09.225]

37 **Su Y**, Simmen RC. Soy isoflavone genistein upregulates epithelial adhesion molecule E-cadherin expression and attenuates beta-catenin signaling in mammary epithelial cells. *Carcinogenesis* 2009; **30**: 331-339 [PMID: 19073877 DOI: 10.1093/carcin/bgn279]

P- Reviewer: Cong WM **S- Editor:** Wen LL
L- Editor: Cant MR **E- Editor:** Ma S

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v19.i43.7696

World J Gastroenterol 2013 November 21; 19(43): 7696-7700
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Laparoscopy-assisted percutaneous endoscopic gastrostomy enables enteral nutrition even in patients with distorted anatomy

Adam Hermanowicz, Ewa Matuszczak, Marta Komarowska, Elzbieta Jarocka-Cyrta, Jerzy Wojnar, Wojciech Debek, Konrad Matysiak, Stanislaw Klek

Adam Hermanowicz, Ewa Matuszczak, Marta Komarowska, Elzbieta Jarocka-Cyrta, Jerzy Wojnar, Wojciech Debek, Department of Pediatric Surgery, Medical University of Bialystok, 15-001 Bialystok, Poland
Konrad Matysiak, Department of Gastroenterology and Oncology and Plastic Surgery, Medical University of Poznan, 60-015 Poznań, Poland
Stanislaw Klek, General and Oncology Surgery Unit, Stanley Dudrick's Memorial Hospital, 32-050 Skawina, Poland
Author contributions: Hermanowicz A and Klek S coordinated the trial, they were responsible for the study conception and contributed to the experimental design, data interpretation and writing of the manuscript; all the authors critically revised the intellectual content of the study and contributed to the writing of the manuscript, the data and statistical analyses and the data collection.
Correspondence to: Stanislaw Klek MD, PhD, Professor, General and Oncology Surgery Unit, Stanley Dudrick's Memorial Hospital, 15 Tyniecka Street, 32-050 Skawina, Poland. klek@poczta.onet.pl
Telephone: +48-12-4248007 Fax: +48-12-4248007
Received: August 15, 2013 Revised: September 19, 2013
Accepted: September 29, 2013
Published online: November 21, 2013

Abstract

AIM: To analyzed whether laparoscopy-assisted percutaneous endoscopic gastrostomy (PEG) could be a valuable option for patients with complicated anatomy.

METHODS: A retrospective analysis of twelve patients (seven females, five males; six children, six young adults; mean age 19.2 years) with cerebral palsy, spastic quadriparesis, severe kyphoscoliosis and interposed organs and who required enteral nutrition (EN) due to starvation was performed. For all patients, standard PEG placement was impossible due to distorted anatomy. All the patients qualified for the laparoscopy-assisted PEG procedure.

RESULTS: In all twelve patients, the laparoscopy-assisted PEG was successful, and EN was introduced four to six hours after the PEG placement. There were no complications in the perioperative period, either technical or metabolic. All the patients were discharged from the hospital and were then effectively fed using bolus methods.

CONCLUSION: Laparoscopy-assisted PEG should become the method of choice for gastrostomy tube placement and subsequent EN if PEG placement cannot be performed safely.

Key words: Percutaneous endoscopic gastrostomy; Laparoscopy-assisted percutaneous endoscopic gastrostomy; Severe kyphosis; Malnutrition; Interposed organs

Core tip: Enteral nutrition (EN) is a life-saving procedure, preventing complications associated with malnutrition. The best solution for EN is percutaneous endoscopic gastrostomy (PEG). In some cases, however, creating such access is impossible. In those cases, laparoscopy-assisted PEG should become the method of choice for gastrostomy tube placement and subsequent EN if PEG placement cannot be performed safely.

Hermanowicz A, Matuszczak E, Komarowska M, Jarocka-Cyrta E, Wojnar J, Debek W, Matysiak K, Klek S. Laparoscopy-assisted percutaneous endoscopic gastrostomy enables enteral nutrition even in patients with distorted anatomy. *World J Gastroenterol* 2013; 19(43): 7696-7700 Available from: URL:

http://www.wjgnet.com/1007-9327/full/v19/i43/7696.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7696

INTRODUCTION

Tube enteral feeding is the method of choice when an oral diet is insufficient or impossible for more than seven days, even in patients without apparent malnutrition[1]. Tube enteral feeding is also strongly recommended if the latter is present or imminent[1]. All these patients should be administered enteral nutrition (EN), and when the oral diet cannot be continued, tube feeding is the best option. Since its introduction by Gauderer in 1980, percutaneous endoscopic gastrostomy (PEG) has become the method of choice for EN in all age groups[2]. The primary goal of EN is to improve the patient's well-being by preventing or reversing malnutrition and avoiding its consequences. EN is particularly important in pediatric patients because they need to not only survive but also grow. Neurological disorders place children at a very high risk of malnutrition; hence, this group of patients benefits very quickly from EN. Unfortunately, neurological disorders are very often accompanied by serious distortions in body anatomy.

Problems, such as severe kyphoscoliosis, interposed organs or other forms of distorted anatomy, may prevent effective and safe PEG placement due to an inability to guarantee the three principles of safe PEG placement: endoscopic gastric distension, endoscopically visible focal finger invagination and transillumination[3,4]. In such cases, surgical gastrostomy, which is an invasive procedure, is often the only option for EN. However, less invasive measures would be preferable over surgical gastronomy. Our study aimed to determine whether laparoscopy-assisted PEG placement is a useful option for EN in patients with distorted anatomy and who are unable to undergo PEG placement.

MATERIALS AND METHODS

This study was a retrospective analysis of twelve patients (seven females, five males; mean age 19.2 years), who were treated at the pediatric surgery center in Bialystok, Poznan and Skawina, Poland. Six patients were children, and the remaining six were adults. In all patients, malnutrition requiring nutritional support was diagnosed (body mass index < 14 m^2/kg), and tube feeding was recognized as the method of choice for EN. In all four patients, PEG placement was impossible due to spastic quadriparesis, severe kyphoscoliosis and interposed organs. Informed consent was collected from all patients. The patients and procedure characteristics are presented in Table 1.

Technique

PEG tube insertion was performed under general anesthesia. A single dose of intravenous antibiotics was given. The patient was positioned in the supine position. After sterilizing the skin on the anterior abdominal wall, a 5-mm port was inserted under the umbilicus using Hasson's open technique[5]. Pneumoperitoneum was established *via* a trocar, using carbon dioxide. The intra-abdominal pressure in our patients was 10 mmHg. The peritoneal cavity and the abdominal and gastric walls were inspected for suitable sites for the gastrostomy. After insertion of the gastroscope into the stomach and air insufflation, the gastrostomy site was selected under laparoscopic and endoscopic guidance. The skin was incised above the gastrostomy site with a length of 0.5 cm. A trocar with a needle was pushed through this point into the stomach under complete laparoscopic and endoscopic visualization. The PEG was made using the 'pull' technique: a thread was inserted through the trocar after removing the needle and was then snared. The endoscope was withdrawn by the snare holding the thread. A suitably sized PEG tube was then connected to the thread, and the thread was pulled from the skin incision, pulling the tube into patient's mouth through the esophagus. The PEG tube was retained in the stomach with an internal bolster. An external bolster was placed loosely on the skin[2,3]. All the procedures were uneventful and without any intraoperative complications.

RESULTS

The laparoscopy-assisted PEG insertion was successful in all twelve patients. The mean length of the procedure was 16.5 min. A Flocare PEG tube (Nutricia Ltd., Poland) with a diameter of 14 Cherrier was used as the gastric catheter.

No postoperative complications were observed, and EN was commenced four to six hours after the PEG placement using the bolus method (5 mL × 100 mL). The mean length of the hospital stay was 1.5 d. All the patients were discharged from the hospital and then effectively fed using bolus methods at long-term facilities. The follow-up at twelve months did not reveal any complications. The nutritional status of the patients improved significantly, with the mean BMI reaching 17.5 m^2/kg (Table 1).

DISCUSSION

Tube feeding is the method of choice when EN is recommended[1]. In most cases, PEG tube insertion is a safe procedure and does not lead to complications[3,6]. Fatal outcomes have been reported due to comorbidities when the PEG was inserted in the setting of severe disease[3,7]. However, the safe placement of a PEG tube requires a permeable esophagus and transillumination of the stomach through the abdominal wall. Impaired coagulation, severe ascites, peritonitis and local esophageal and general gastrointestinal obstructions are considered to be absolute contraindications[3,4,8]. Severe kyphoscoliosis with interposed organs and distorted anatomy are

Table 1 Patients' characteristics, the reasons for L-percutaneous endoscopic gastrostomy, the length of the procedures, the nutritional statuses before and after L-percutaneous endoscopic gastrostomy

No.	Gender	Age (yr)	Reason of LAPEG	BMI before the procedure/ albumin concentration (g/dL)	BMI after 12 mo follow up	Time from insertion of the gastroscope to the peg placement (total operation length)/min	Time form insertion laparoscope to removal of the laparoscope/min
1	F	7	Tay-Sachs disease	11.9/2.9	14.5	22	16
2	M	5	drug-resistant epilepsy	13.9/3.9	15.2	17	13
3	M	15	cerebral palsy	13.2/4.5	15.2	15	9
4	F	3	Patau syndrome	13.8/5.1	16.0	21	12
5	M	17	cerebral palsy	12.6/3.3	18.4	24	14
6	M	11	cerebral palsy	13.2/3.7	17.1	22	9
7	F	22	Wilson's disease	12.0/28	13.5	21	17
8	F	31.5	Gaucher's disease	14.5/29.5	14.5	18	12
9	M	26.2	cerebral palsy	15.5/27.0	15.2	16	8
10	F	33.2	SLA	14.0/25.0	17.0	12	11
11	F	34	SLA	11.0/2.3	14.5	14	11
12	F	24.7	cerebral palsy	11.5/2.9	15.0	13	7

BMI: Body mass index; LAPEG: LA-percutaneous endoscopic gastrostomy; SLA: Amyothropic later sclerosis; M: Male; F: Female.

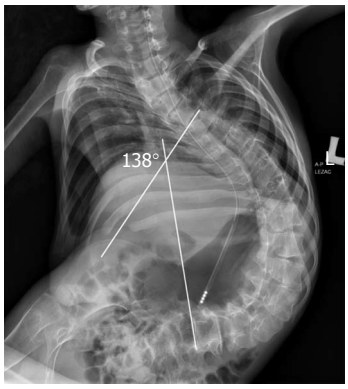


Figure 1 A 17-year-old patient with severe kyphoscoliosis (138 degrees on Cobb's scale) with interposed organs.

considered to be relative contraindications[8-10] (Figure 1). In patients with severe kyphoscoliosis, the PEG tube cannot be placed in the usual locations due to organ displacement. In our patients, the contraindications for PEG included the translocation of the stomach into the left lower quadrant of the abdomen (3 patients) or into the middle part of the abdomen (1 patient) (Figure 2). Therefore, in patients with severe kyphoscoliosis, patients suffering from hepatomegaly, splenomegaly, obesity or an intra-abdominal or peritoneal tumor, patients with previous abdominal surgery, especially involving the stomach, and patients with advanced esophageal cancer, when transillumination of the stomach is not achieved, there is an indication for laparoscopic, fluoroscopic or sonographic guidance during the PEG tube placement.

The technique for the laparoscopic-assisted PEG procedure was described for the first time by Raaf *et al*[11] in 1993. In 1995, Stringel *et al*[12] reported a laparoscopic-assisted PEG procedure in 2 children in whom attempts at a simple PEG had failed. There are also other alternative methods for PEG placement when transillumination is not possible. Radiologic techniques have been used successfully in these patients since 1981[13,14]. The gastric tubes can be placed under ultrasound or fluoroscopic guidance[15-17]. Recently, the placement of the gastric tubes has used Computed tomography (CT) fluoroscopy guidance, even in combination with simultaneous gastroscopy[18].

Historically, gastroesophageal reflux was also considered to be a contraindication, as concluded by Yaseen *et al*[19]. Recent trials have shown that gastroesophageal reflux might actually improve after PEG placement because the PEG creates a type of anterior pseudo-gastropexy[20,21].

Major complications related to PEG include the following: colonic perforation, esophageal tear, small bowel injury, hepatic or splenic injury, tube migration with or without intestinal obstruction, gastrointestinal bleeding and site or generalized infection. These complications have been reported with variable incidences (5%-17%) in published series[3,4,7,22]. The most severe complication, with an incidence of 0.0008%-0.04%, is esophageal perforation[4,23]. Predisposing factors include anatomic anomalies in up to 50% of cases[3]. Patients with displacement of the transverse colon over the anterior gastric wall are predisposed to colonic injury during the PEG procedure[3,23,24]. Colonic injury usually presents with peritonitis, and surgery is then required. Additionally, interposition of the splenic flexure between the anterior abdominal and gastric walls may result in gastro-colo-cutaneous fistulae after PEG placement without direct visual monitoring. The PEG tube is placed through the large bowel into the stomach. Such patients may be almost completely asymptomatic except for transient fever and ileus. The management involves PEG tube removal and spontaneous closure of the fistula[4,25]. Small bowel injuries are rather rare because the greater omentum separates the small bowel from the upper abdomen. Small bowel volvulus around the PEG usually presents with a small bowel obstruction caused by a gap between the gastric and abdominal walls[4]. Hepatic and splenic PEG-related injuries are also rare. In hemodynamically stable patients, a CT scan can confirm the diagnosis. Hemodynamically unstable patients require emergent surgical exploration, placement of hemostatic *sutures* in the

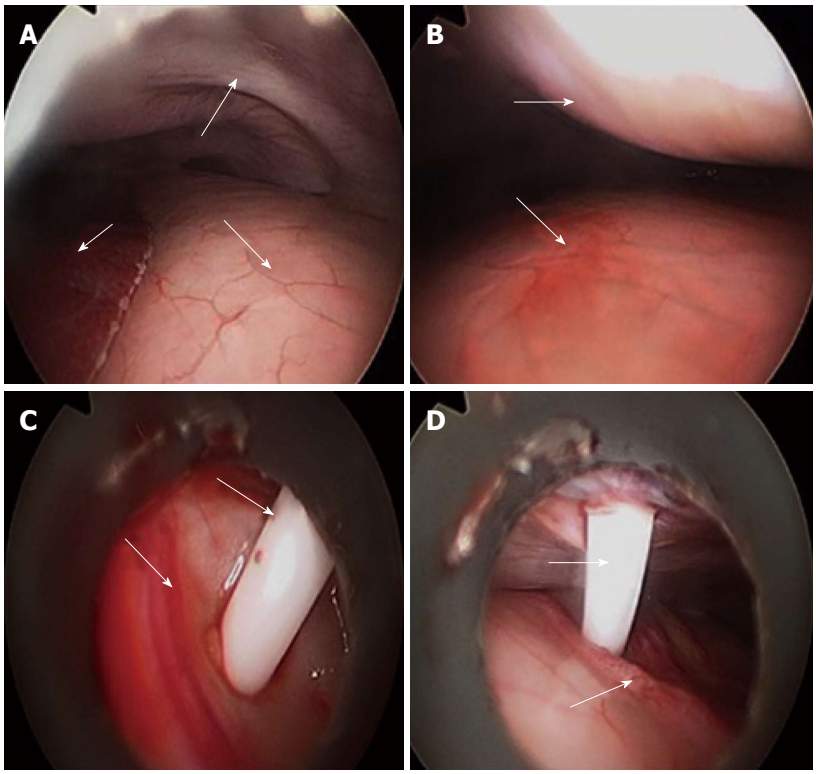


Figure 2 Laparoscopy-assisted percutaneous endoscopic gastrostomy placement: An overview of the procedure. A: General view. The arrows indicate the following (respectively): liver, stomach and abdominal wall; B: Marking the puncture site. The arrows indicate the stomach and abdominal walls, and the pressed wall shows the puncture site; C: Introduction of the needle into the stomach under visual control. The arrows show the gastric wall and the trocar for percutaneous endoscopic gastrostomy (PEG) insertion; D: PEG was performed. The arrows indicate the gastric vessels and the trocar.

liver or a splenectomy. Severe hemorrhage is a rare complication of PEG (0.02%-0.06%) and is usually due to anticoagulation, antiplatelet therapy or an anatomic anomaly, as observed in our patients[3,23]. We did not notice any major complications from the PEG tube insertion in our patients. In our opinion, only continuous laparoscopic monitoring can ensure that the omentum, colon and small bowel will not be interposed between the stomach and the anterior abdominal wall during gastrostomy fixation in extremely malnourished children with severe kyphoscoliosis.

Minor complications, such as superficial skin infections, superficial granulation tissue formation and tube leak, are common and may occur in up to 50% of patients[3,4,9,26]. Minor skin infections usually respond to enteral antibiotics, and granulation tissue usually responds to local cautery with silver nitrate swabs. We did not notice any skin infections, tube leaks or granulation tissue formation in our patients. The patients' families and caregivers were taught how to flush the tube after feeding to avoid tube obstruction.

With respect to the laparoscopy, we use Hasson's open method and an optical access trocar to achieve pneumoperitoneum[5]. In our opinion, this method decreases the risk of injuries compared with the blind insertion of the Veress needle; however, Hasson's method may cause air leaks and prolong the operative time. In our cases, the mean duration of the operation time from the insertion of the laparoscope to the removal of the laparoscope did not differ significantly from that of the classic PEG procedure. We did not have continuous air leaks, and the operating time was not prolonged much because of the laparoscopy. Therefore, we have continued to use this technique as the preferred method for inducing pneumoperitoneum in children. Under laparoscopic observation, the stomach can be punctured in the correct location, avoiding the colon or the liver on its way into the gastric lumen. In our study, the laparoscopic-assisted PEG procedures were performed without difficulty. There was no need to maneuver or relocate the interposed organs during the laparoscopy. This procedure, for which the technical aspects are presented in Figure 2, allowed for successful EN. The subsequent EN helped our patients recover from starvation and decreased their malnutrition-related complication ratio.

In conclusion, the laparoscopy-assisted PEG procedure is a valuable method for gastrostomy tube placement in patients in whom an upper endoscopy is possible but PEG cannot be performed safely. In our opinion, laparoscopy-assisted PEG should become the method of choice for children and adults with distorted anatomy.

COMMENTS

Background

Enteral nutrition (EN) is a life-saving procedure that prevents the complications associated with malnutrition. Percutaneous endoscopic gastrostomy (PEG) is the method of choice for this type of intervention because the procedure en-

ables tube feeding.

Research frontiers

PEG placement is not always possible due to technical difficulties, such as interposed organs or kyphosis. This study aimed to analyze whether laparoscopy-assisted PEG could be a valuable option in those patients in whom the standard procedure is impossible due to distorted anatomy.

Innovations and breakthroughs

For all the patients, the laparoscopy-assisted PEG tube placement was successful, and the EN was started four to six hours after its placement, proving that this technique is safe and effective.

Applications

Laparoscopy-assisted PEG should become the method of choice for gastrostomy tube placement and subsequent EN if PEG placement cannot be performed safely.

Peer review

This is an interesting article of case series of laparoscopy-assisted PEG.

REFERENCES

1 **Weimann A**, Braga M, Harsanyi L, Laviano A, Ljungqvist O, Soeters P, Jauch KW, Kemen M, Hiesmayr JM, Horbach T, Kuse ER, Vestweber KH. ESPEN Guidelines on Enteral Nutrition: Surgery including organ transplantation. *Clin Nutr* 2006; **25**: 224-244 [PMID: 16698152 DOI: 10.1016/j.clnu.2006.01.015]

2 **Gauderer MW**. Percutaneous endoscopic gastrostomy and the evolution of contemporary long-term enteral access. *Clin Nutr* 2002; **21**: 103-110 [PMID: 12056781 DOI: 10.1054/clnu.2001.0533]

3 **El-Matary W**. Percutaneous endoscopic gastrostomy in children. *Can J Gastroenterol* 2008; **22**: 993-998 [PMID: 19096739]

4 **Strassler HE**. A combination of dental procedures gives patients the desired smile. *Dent Off* 1991; **11**: 4, 7 [PMID: 1819312]

5 **Humphrey GM**, Najmaldin A. Modification of the Hasson technique in paediatric laparoscopy. *Br J Surg* 1994; **81**: 1319 [PMID: 7953399 DOI: 10.1002/bjs.1800810920]

6 **Catto-Smith AG**, Jimenez S. Morbidity and mortality after percutaneous endoscopic gastrostomy in children with neurological disability. *J Gastroenterol Hepatol* 2006; **21**: 734-738 [PMID: 16677161 DOI: 10.1111/j.1440-1746.2005.03993.x]

7 **Gauderer MW**. Percutaneous endoscopic gastrostomy: a 10-year experience with 220 children. *J Pediatr Surg* 1991; **26**: 288-292; discussion 292-294 [PMID: 2030474 DOI: 10.1016/0022-3468(91)90504-M]

8 **Löser C**, Aschl G, Hébuterne X, Mathus-Vliegen EM, Muscaritoli M, Niv Y, Rollins H, Singer P, Skelly RH. ESPEN guidelines on artificial enteral nutrition--percutaneous endoscopic gastrostomy (PEG). *Clin Nutr* 2005; **24**: 848-861 [PMID: 16261664 DOI: 10.1016/j.clnu.2005.06.013]

9 **DeLegge RL**, DeLegge MH. Percutaneous endoscopic gastrostomy evaluation of device materials: are we "failsafe"? *Nutr Clin Pract* 2005; **20**: 613-617 [PMID: 16306298 DOI: 10.1177/0115426505020006613]

10 **Broscious SK**. Preventing complications of PEG tubes. *Dimens Crit Care Nurs* 1995; **14**: 37-41 [PMID: 7743905 DOI: 10.1097/00003465-199501000-00007]

11 **Raaf JH**, Manney M, Okafor E, Gray L, Chari V. Laparoscopic placement of a percutaneous endoscopic gastrostomy (PEG) feeding tube. *J Laparoendosc Surg* 1993; **3**: 411-414 [PMID: 8268516 DOI: 10.1089/lps.1993.3.411]

12 **Stringel G**, Geller ER, Lowenheim MS. Laparoscopic-assisted percutaneous endoscopic gastrostomy. *J Pediatr Surg* 1995; **30**: 1209-1210 [PMID: 7472985 DOI: 10.1016/0022-3468(95)90024-1]

13 **Preshaw RM**. A percutaneous method for inserting a feeding gastrostomy tube. *Surg Gynecol Obstet* 1981; **152**: 658-660 [PMID: 6784260]

14 **Lim A Po IF**, van Overhagen H, Nicolai JJ. [Gastrostomy tubes inserted with radiologic techniques]. *Ned Tijdschr Geneeskd* 2003; **147**: 373-377 [PMID: 12661453]

15 **Hoffer EK**. US-guided percutaneous gastrostomy: a portable technique. *J Vasc Interv Radiol* 1996; **7**: 431-434 [PMID: 8761826 DOI: 10.1016/S1051-0443(96)72883-X]

16 **Ahlers CM**, Schneider J, Lachmann R, Herber S, Düber C, Pitton MB. Fluoroscopy-guided removal of pull-type gastrostomy tubes. *Cardiovasc Intervent Radiol* 2008; **31**: 1252-1254 [PMID: 18418649 DOI: 10.1007/s00270-008-9335-3]

17 **Chaves DM**, Kumar A, Lera ME, Maluf F, Artifon EL, Moura EG, Halwan B, Ishioka S, Sakai P. EUS-guided percutaneous endoscopic gastrostomy for enteral feeding tube placement. *Gastrointest Endosc* 2008; **68**: 1168-1172 [PMID: 19028225 DOI: 10.1016/j.gie.2008.06.062]

18 **Spelsberg FW**, Hoffmann RT, Lang RA, Winter H, Weidenhagen R, Reiser M, Jauch KW, Trumm C. CT fluoroscopy guided percutaneous gastrostomy or jejunostomy without (CT-PG/PJ) or with simultaneous endoscopy (CT-PEG/PEJ) in otherwise untreatable patients. *Surg Endosc* 2013; **27**: 1186-1195 [PMID: 23232989 DOI: 10.1007/s00464-012-2574-z]

19 **Yaseen M**, Steele MI, Grunow JE. Nonendoscopic removal of percutaneous endoscopic gastrostomy tubes: morbidity and mortality in children. *Gastrointest Endosc* 1996; **44**: 235-238 [PMID: 8885339 DOI: 10.1016/S0016-5107(96)70157-8]

20 **Wilson GJ**, van der Zee DC, Bax NM. Endoscopic gastrostomy placement in the child with gastroesophageal reflux: is concomitant antireflux surgery indicated? *J Pediatr Surg* 2006; **41**: 1441-1445 [PMID: 16863851 DOI: 10.1016/j.jpedsurg.2006.04.021]

21 **Samuel M**, Holmes K. Quantitative and qualitative analysis of gastroesophageal reflux after percutaneous endoscopic gastrostomy. *J Pediatr Surg* 2002; **37**: 256-261 [PMID: 11819210 DOI: 10.1053/jpsu.2002.30267]

22 **Khattak IU**, Kimber C, Kiely EM, Spitz L. Percutaneous endoscopic gastrostomy in paediatric practice: complications and outcome. *J Pediatr Surg* 1998; **33**: 67-72 [PMID: 9473103 DOI: 10.1016/S0022-3468(98)90364-5]

23 **Gonvers JJ**, Burnand B, Froehlich F, Pache I, Thorens J, Fried M, Kosecoff J, Vader JP, Brook RH. Appropriateness and diagnostic yield of upper gastrointestinal endoscopy in an open-access endoscopy unit. *Endoscopy* 1996; **28**: 661-666 [PMID: 8934082 DOI: 10.1055/s-2007-1005573]

24 **Guloglu R**, Taviloglu K, Alimoglu O. Colon injury following percutaneous endoscopic gastrostomy tube insertion. *J Laparoendosc Adv Surg Tech A* 2003; **13**: 69-72 [PMID: 12676027 DOI: 10.1089/109264203321235520]

25 **Berger SA**, Zarling EJ. Colocutaneous fistula following migration of PEG tube. *Gastrointest Endosc* 1991; **37**: 86-88 [PMID: 1900799 DOI: 10.1016/S0016-5107(91)70634-2]

26 **Ségal D**, Michaud L, Guimber D, Ganga-Zandzou PS, Turck D, Gottrand F. Late-onset complications of percutaneous endoscopic gastrostomy in children. *J Pediatr Gastroenterol Nutr* 2001; **33**: 495-500 [PMID: 11698770 DOI: 10.1097/00005176-200110000-00015]

P- Reviewers: Li XL, Nishiwaki S, Radaelli F
S- Editor: Qi Y **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.v19.i43.7701

World J Gastroenterol 2013 November 21; 19(43): 7701-7710
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Is early limited surgery associated with a more benign disease course in Crohn's disease?

Petra Anna Golovics, Laszlo Lakatos, Attila Nagy, Tunde Pandur, Istvan Szita, Mihaly Balogh, Csaba Molnar, Erzsebet Komaromi, Barbara Dorottya Lovasz, Michael Mandel, Gabor Veres, Lajos S Kiss, Zsuzsanna Vegh, Peter Laszlo Lakatos

Petra Anna Golovics, Barbara Dorottya Lovasz, Michael Mandel, Lajos Sandor Kiss, Zsuzsanna Vegh, Peter Laszlo Lakatos, First Department of Medicine, Semmelweis University, H-1083 Budapest, Hungary
Laszlo Lakatos, Tunde Pandur, Istvan Szita, Department of Medicine, Csolnoky F Province Hospital, H-8200 Veszprem, Hungary
Attila Nagy, Department of Surgery, Csolnoky F Province Hospital, H-8200 Veszprem, Hungary
Mihaly Balogh, Department of Medicine, Grof Eszterhazy Hospital, H-8500 Papa, Hungary
Csaba Molnar, Department of Infectious Diseases, Magyar Imre Hospital, H-8400 Ajka, Hungary
Erzsebet Komaromi, Department of Gastroenterology, Municipal Hospital, H-8100 Varpalota, Hungary
Gabor Veres, First Department of Pediatrics, Semmelweis University, H-1083 Budapest, Hungary
Author contributions: Golovics PA, Lakatos L, Lovasz BD and Vegh Z contributed to data collection and validation, database construction, and manuscript preparation and revision; Nagy A, Pandur T, Szita I, Balogh M, Molnar C and Komaromi E contributed to data collection and critical revision of the manuscript; Mandel M, Veres G and Kiss LS contributed to data validation and critical revision of the manuscript; Lakatos PL contributed tostudy design, data collection and validation, database construction, statistical analysis, and manuscript preparation; all authors have approved the final draft submitted.
Supported by Unrestricted research grant by Schering-Plough Hungary/MSD to Lakatos PL and Lakatos L
Correspondence to: Peter Laszlo Lakatos, MD, PhD, First Department of Medicine, Semmelweis University, Koranyi S. 2/A, H-1083 Budapest,
Hungary. lakatos.peter_laszlo@med.semmelweis-univ.hu
Telephone: +36-1-4591500 Fax: +36-1-3130250
Received: July 1, 2013 Revised: August 2, 2013
Accepted: September 13, 2013
Published online: November 21, 2013

Abstract

AIM: To analyze the difference in disease course and need for surgery in patients with Crohn's disease (CD).

METHODS: Data of 506 patients with incident CD were analyzed (age at diagnosis: 31.5 ± 13.8 years). Both hospital and outpatient records were collected prospectively with a complete clinical follow-up and comprehensively reviewed in the population-based Veszprem province database, which includes incident CD patients diagnosed between January 1, 1977 and December 31, 2008. Follow-up data were collected until December 31, 2009. All patients included had at least 1 year of follow-up available. Patients with indeterminate colitis at diagnosis were excluded from the analysis.

RESULTS: Overall, 73 patients (14.4%) required resective surgery within 1 year of diagnosis. Steroid exposure and need for biological therapy were lower in patients with early limited surgery ($P < 0.001$ and $P = 0.09$). In addition, surgery rates during follow-up in patients with and without early surgery differed significantly after matching on propensity scores ($P < 0.001$, HR = 0.23). The need for reoperation was also lower in patients with early limited resective surgery ($P = 0.038$, HR = 0.42) in a Kaplan-Meier and multivariate Cox regression ($P = 0.04$) analysis. However, this advantage was not observed after matching on propensity scores ($P_{Logrank} = 0.656$, $P_{Breslow} = 0.498$).

CONCLUSION: Long-term surgery rates and overall exposure to steroids and biological agents were lower in patients with early limited resective surgery, but reoperation rates did not differ.

Key words: Crohn's disease; Early surgery; Disease course; Disease behavior; Treatment strategy

Core tip: An alternative approach may be early limited

resective surgery in a well-selected group of patients with Crohn's disease. In this population-based study, we found that overall exposure to steroids and biological agents was lower in patients with early limited resective surgery; observed surgery rates were also lower, yet reoperation rates did not differ in the two groups after matching on propensity scores.

Golovics PA, Lakatos L, Nagy A, Pandur T, Szita I, Balogh M, Molnar C, Komaromi E, Lovasz BD, Mandel M, Veres G, Kiss LS, Vegh Z, Lakatos PL. Is early limited surgery associated with a more benign disease course in Crohn's disease? *World J Gastroenterol* 2013; 19(43): 7701-7710 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7701.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7701

INTRODUCTION

Crohn's disease (CD) has a variable course, but the majority of patients eventually develop penetrating or stricturing complications. In addition, several environmental risk factors (diet, smoking, measles, or appendectomy) may contribute to its etiology and course. A significant adverse outcome is the need for surgery. Nevertheless, surgery is not curative in CD. Surgical resection is typically performed for emergency indications (*e.g.*, obstructive symptoms and hemorrhage) or for failure to respond to medical therapy.

Some years ago, a review article reported that the probability of first resective surgery ranged from 38% to 96% in the first 15 years after diagnosis[1]. The overall clinical relapse and reoperation rates after initial resective surgery are 50%-60% and 28%-45%, respectively, during the following 15 years. Surgical resection rates over time vary widely among published studies, ranging between 25 and 61% in the first 5 years. Until recently, there was little evidence that disease outcomes for CD had changed over recent decades. Recently, Peyrin-Biroulet *et al*[2] published a systematic review of the natural history of CD in population-based cohorts. According to the authors' conclusions, the impact of changing treatment paradigms with the increased use of immunosuppressants and biological agents on the natural history of the disease was poorly understood. Available data did not suggest a significant change in outcome of CD, with approximately half of patients requiring surgery within 10 years of diagnosis. The risk of postoperative clinical recurrence within 10 years was 44%-55%.

A recent meta-analysis from International Organization for the Study of Inflammatory Bowel Diseases (IOIBD) Epidemiology Task Force reported that the risk of surgery in CD in prebiologic population-based cohorts has been decreasing during the past decade[3]. One of the most striking changes was reported by Jess *et al*[4] in a Danish study. The rate of early surgery (within 1 year of diagnosis) has fallen from 35% (1962-1987) to 12% (2003-2004). During this time, there was a significant change in patient management; namely, increased and earlier use of immunosuppressants and the introduction of biological therapies. The effect of azathioprine (AZA) on disease prognosis was until recently controversial. Two recent population-based reports confirmed that early AZA use is associated with reduced need for surgery according to a Cox regression analysis and propensity score matching in two population-based cohorts from Wales and Hungary[5,6]. Furthermore, in a study from France, an association was reported between the duration of anti-tumor necrosis factor (TNF) and AZA therapy and risk for surgery[7]. In contrast, in a previous referral center study from France, the need for intestinal surgery did not decrease despite the increased use of immunosuppressants[8]. Of note, in this study, AZA therapy was started only after surgery in the majority of patients. Earlier AZA use is only one of the complex changes in patient management. Other changes have also occurred, including a trend toward tight patient monitoring. Moreover, whether the risk of surgery is affected by the more widespread use of biological agents has yet to be demonstrated by population-based studies.

An alternative approach to the predominant strategy of initially using conservative therapy: using limited resective surgery in a selected group of patients as a primary therapeutic option, may prove advantageous. In a study by Aratari *et al*[9], early surgery at diagnosis in 207 CD patients with ileocecal disease was associated with a more benign postoperative disease course, in comparison to patients receiving delayed surgery. Nevertheless, reoperation rates were not reduced. Thus, in a subgroup of CD patients, early surgery may represent a valid alternative to medical therapy; particularly in patients with limited, isolated, stenotic ileocecal disease.

Therefore, our aim was to analyze the disease course, drug exposure and need for surgery and reoperation in patients with and without early (within 1 year of diagnosis) limited resective surgery in a population-based cohort from Eastern Europe with a complete clinical follow-up.

MATERIALS AND METHODS

Patients

A well-characterized Hungarian cohort of 506 patients with incident CD (male/female: 251/255; age at diagnosis: 31.5 ± 13.8 years) diagnosed between January 1, 1977 and December 31, 2008 were included. Follow-up data were collected until December 31, 2009. All patients included had at least 1 year follow-up available. Patients with indeterminate colitis at diagnosis were excluded from the analysis. The clinical data of CD patients are summarized in Table 1.

Methods

Clinical data were collected every year from the seven general hospitals (departments of internal medicine, surgery, and pediatrics) and gastroenterology outpatient units. The majority of patients [76% of ulcerative colitis (UC) and

94% of CD patients] were monitored at the Csolnoky F. Province Hospital in Veszprem, where data were also registered. Disease behavior was updated yearly. A more detailed description of the data collection method, case assessment, the geographical and socioeconomic background of the province, and the results of surgical and medical management, as well as a detailed description of the Veszprem Province inflammatory bowel disease (IBD) Group, was published in previous epidemiological studies[10-12].

The disease phenotype (age at onset, duration, location, and behavior) was determined according to the Montreal Classification[13]. The presence of perianal disease and behavior change during follow-up were also registered. Medical therapy was recorded in detail (as defined by the European Crohn's and Colitis Organisation Consensus Report[14]). The need for surgery or reoperation and smoking habits were investigated by review of medical files and by questionnaire.

The study was approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics and by the Csolnoky F. Province Hospital Institutional Committee of Science and Research Ethics.

Treatment policy

The majority of the patients received maintenance therapy with sulfasalazine or a 5-aminosalicylic acid derivative (mesalazine or olsalazine), if tolerated, especially until the mid-1990s. AZA or 6-mercaptopurine (6-MP) was used in selected cases as maintenance therapy for steroid-dependent and -refractory patients or for patients with fistulizing disease. AZA and 6-MP were typically used following resective surgery until the late 1980s, and later on a more widespread basis beginning in the mid-1990s. Short-term oral corticosteroid treatment was used for clinical exacerbations, usually prednisone 40-60 mg/d, tapered and discontinued over the course of 2-3 mo. Beginning in the mid-1990s, methotrexate was used as second-line immunosuppressive therapy in limited cases. Since the late 1990s, infliximab has been used for both induction and maintenance therapy in selected cases.

Surgical resection was performed for emergency indications (*e.g.*, obstructive symptoms and hemorrhage) and for failure to respond to medical therapy. Surgical techniques have also changed during the follow-up period of this study; laparoscopic surgery became available and more widely used from the late 1990s. Limited resections were more widely used from the mid-1990s than in the past, and there is very limited use of defunctioning ileostomy (n = 4) formation in routine management of CD. For the majority of patients in the present study, one of the most experienced IBD surgeons in Hungary performed the operations, while laparoscopic surgery and stricturoplasty were performed only in a minority of cases. The definition of early limited surgery was resection of the terminal ileum or ileocecum within 1 year of diagnosis.

Due to Hungarian health authority regulations, a follow-up visit is obligatory for IBD patients at a specialized gastroenterology center every 6 mo. Otherwise, under the regulations of the Hungarian National Health Insurance (OEP) system, the right to subsidized therapy is forfeited. Consequently, the relationship between IBD patients and specialists is a close one.

Propensity score model

The authors used two propensity score models to control further for possible confounders, to quantify the probability of surgery and reoperation in patients who had early limited resective surgery versus those who did not.

In the first propensity model, covariates included in the propensity score were selected according to a two-step process. We first constructed an outcome model identifying independent predictors of early limited resective surgery. Subsequently, we included in the model additional predictors known to be associated with surgical outcome (*e.g.*, age at onset and smoking) irrespective of P value. We used multivariate logistic regression to estimate propensity scores of early limited resection for each individual. Goodness-of-fit was evaluated by the Hosmer-Lemeshow test, the P values of which were not significant. Using the predicted propensity scores from our model, we attempted to match all early limited surgery to identical CD patients without early limited surgery by 5-to-1 greedy matching[15]. Additional analysis in patients with ileum-only disease was also performed.

In the second propensity model, we aimed to analyze the probability of reoperation in patients with and without early surgery in a group of CD patients with a history of at least one resective operation. Using the above two-step process and predicted propensity scores from our model, we attempted to match all early surgery CD patients to identical CD patients with a non-early surgical resection through 5-to-1 greedy matching[15].

Ethical permission

The study protocol was approved by Semmelweis University Regional and Institutional Committee of Science and Research Ethics and the Csolnoky F. Province Hospital Institutional Committee of Science and Research Ethics

Statistical analysis

Variables were tested for normality by the Shapiro-Wilk W test. Wilcoxon rank sum test, χ^2 test, and χ^2 test with Yates correction and logistic regression were used to test differences in disease phenotype between subgroups of UC and CD patients for dichotomous variables. Kaplan-Meier survival curves were plotted for analysis with log rank and Breslow tests to determine probability of surgical resection. Additionally, Cox regression analysis using the enter method was used to assess the association between categorical clinical variables and time to AZA use and surgical requirements. Variables with $P < 0.2$ in univariate analysis were included in the multivariate testing. To control further for possible confounders, we developed a propensity score models (see below) for quantifying the probability of reoperation in patients with

Table 1 Clinical characteristics of patients with Crohn's disease

Characteristics	*n* = 506
Male/female	251/255
Age at presentation (yr)	31.5 ± 13.8
Median follow-up (yr)	11.4 ± 7.8
Familial IBD	12.9%
Location (*n*)	
L1	166
L2	182
L3	155
L4 only	3
Behavior (*n*)	
B1	288
At diagnosis B2	100
B3	118
Perianal disease	25.5%
Total steroid exposure/dependent-refractory	68.6%/11.2%
Total azathioprine exposure	45.8%
Total biological exposure	9.1%
Smoking habits (*n*)	
No	224
At diagnosis Ex	38
Yes	244

L1: Ileal; L2: Colonic; L3: Ileocolonic; L4: Upper Gastrointestinal; B1: Inflammatory; B2: Stenosing; B3: Penetrating. IBD: Inflammatory bowel disease.

and without early limited resective surgery[15]. Matching on propensity scores is one technique commonly used to control for measured confounders in observational studies. $P < 0.05$ was considered significant. Results for continuous variables were expressed as median (lower and upper quartile) unless otherwise stated. For the statistical analysis, SPSS version 20.0 was used (SPSS, Chicago, IL, United States).

RESULTS

Patient phenotype

Five hundred and six residents in Veszprem Province were diagnosed with CD in the 32-year period. Follow-up information was collected up to December 31, 2009, equaling 5758 patient-years of follow-up. The clinical characteristics and disease course according to the year of diagnosis is shown in Table 1. There were significant differences in disease phenotype, drug exposure, and smoking habits in the patient groups diagnosed in the early period and thereafter. Overall, exposure to AZA, systemic steroids and biological agents (after 1998 only) was 45.8%, 68.6% and 9.5%, respectively. Total AZA exposure increased in the subsequent cohorts despite shorter follow-up.

Prevalence, predictors of surgery and early limited surgery and drug exposure

A total of 204 (40.7%) patients had at least one resective operation (5 patients with resective surgery due to malignant disease were excluded from analysis). The most common surgical procedures were ileocecal resection (*n* = 93), right hemicolectomy (*n* = 59), segmental colonic resection (*n* = 19), and subtotal colectomy/left hemicolectomy (*n* = 11 and 8, respectively).

Table 2 Predictors of early limited surgery and drug exposures

	Early surgery	No early surgery	*P* value	OR (95%CI)
Age at onset (A1)	28.4%	19.0%	0.060	1.69 (0.97-2.96)
Ileal location	59.5%	28.1%	< 0.001	
Colonic location	13.5%	39.9%	< 0.001	
Complicated behavior at diagnosis	85.1%	35.9%	< 0.001	10.2 (5.2-20.1)
Overall steroid exposure	52.7%	71.3%	0.001	0.45 (0.27-0.74)
Steroid-dependent course	2.6%	12.3%	0.070	0.19 (0.03-1.40
Overall biological exposure	4.1%	10.0%	0.090	0.38 (0.12-1.26)
Overall azathioprine exposure	45.9%	45.8%	> 0.050	

A further 36 (7.1%) patients had other surgical procedures (abscess drainage or fistulectomy). Forty-two (8.4%) patients had two resective operations and 17 (3.4%) had three or more operations for CD during follow-up. Ileocecal resection was the most common procedure overall.

The probability of first intestinal surgery due to non-malignant disease after 1, 5 and 10 years was 14.6%, 30.1%, and 51.6%, respectively, in a Kaplan-Meier analysis. The cumulative probability of resective surgery rate decreased in patients diagnosed in the last decade [Group 1 (1977-1998), Group 2 (1999-2008); $P_{Logrank} = 0.022$, and $P_{Breslow} = 0.07$].

Overall, 73 patients (14.4%) required resective surgery within 1 year of diagnosis. Ten patients were excluded from further analysis from the early surgery group in whom extensive index surgery was performed. The prevalence of early limited resective surgery did not differ significantly in the three cohorts [Cohort A (diagnosed 1977-1989): 11.3%; Cohort B (1990-1998): 12.8%; and Cohort C (1999-2008): 13.4%]. Predictors of early limited resective surgery were ileal location ($P < 0.001$), colonic location ($P < 0.001$), complicated behavior at diagnosis ($P < 0.001$), and age of onset ($P = 0.06$, Table 2).

Overall steroid exposure was significantly lower ($P = 0.001$) in patients with early limited resection despite similar follow-up (median: 12 years). The same trend was observed for steroid dependency ($P = 0.07$) and overall biological agent exposure ($P = 0.09$). In contrast, overall AZA exposure was similar between the two groups. Of note, AZA exposure before surgery was 0% *vs* 28.8% ($P < 0.001$), while TNF-antagonist exposure before surgery was 0% *vs* 4.9% ($P = 0.05$) in patients with and without early limited surgery. In logistic regression analysis, disease location ($P < 0.001$) and disease behavior ($P < 0.001$) were associated with the need for early resective surgery (Table 3).

Table 3 Factors associated with the need for early limited surgery in logistic regression analysis

	P value	OR	95%CI
Age at diagnosis			
A1	0.172	1.62	0.81-3.25
A2		Reference	
Disease location	< 0.001		
L1	< 0.001	7.88	2.92-21.3
L3	0.035	3.21	1.09-9.48
L2		Reference	
Disease behavior at diagnosis	< 0.001		
B2	< 0.001	4.91	2.15-11.2
B3	< 0.001	7.66	3.52-16.7
B1		Reference	
Smoking			
Yes	0.487	1.24	0.68-2.27
No		Reference	

L1: Ileal; L2: Colonic; L3: Ileocolonic; B1: Inflammatory; B2: Stenosing; B3: Penetrating.

Table 4 Characteristics of patients with and without early resective surgery after matching on propensity scores

	CD patient with early surgery (*n* = 58)	CD patient without early surgery (*n* = 58)[1]
Gender (male/female)	32/26	34/24
Age at onset below 40 yr	12	11
Decade of diagnosis		
1977-1989	8	9
1990-1999	22	23
2000-2008	28	26
Disease location		
L1	38	39
L3[2]	20	19
Disease behavior at diagnosis		
B1	10	10
B2	20	19
B3	28	29
Smoking (yes/no)	36/22	35/23

[1]Early resective surgery: within the year of diagnosis; [2]Ileocecal. CD: Crohn's disease; L1: Ileal; L2: Colonic; L3: Ileocolonic; B1: Inflammatory; B2: Stenosing; B3: Penetrating.

Risk of operation in patients with and without early limited surgery

In a propensity score model, we compared surgery rates between patients with and without early resective surgery. In the early limited surgery model, propensity scores ranged from 0.02 to 0.56 (median: 0.29) in patients with early limited resection (n = 63), and from 0.02-0.55 (median: 0.14) in patients without early limited surgery (n = 428). Goodness-of-fit was evaluated by the Hosmer-Lemeshow test, and P values were non-significant (P = 0.653).

Using a 5-to-1 greedy matching algorithm, we were able to match 58 patients from the early resective surgery group to patients with comparable phenotype without early limited resections (Table 4). The observed first resective surgery rate was 12.1%, 33.2%, and 53.6% after 2, 5 and 10 years of disease duration, respectively, in the latter group. In contrast, the reoperation rate in the former group was 1.8%, 5.8%, and 17.9% after 2, 5 and 10 years ($P_{Logrank}$ < 0.001, HR = 0.23, 95%CI: 0.11-0.48; Figure 1A).

If the analysis was restricted to patients with ileum-only location (n = 38), the observed first resective surgery rate was 21%, 35.3%, and 59.4% in patients without early surgery after 2, 5 and 10 years of disease duration, respectively. In contrast, the observed reoperation rate in the other group was 0%, 5.8%, and 20.8% after 2, 5 and

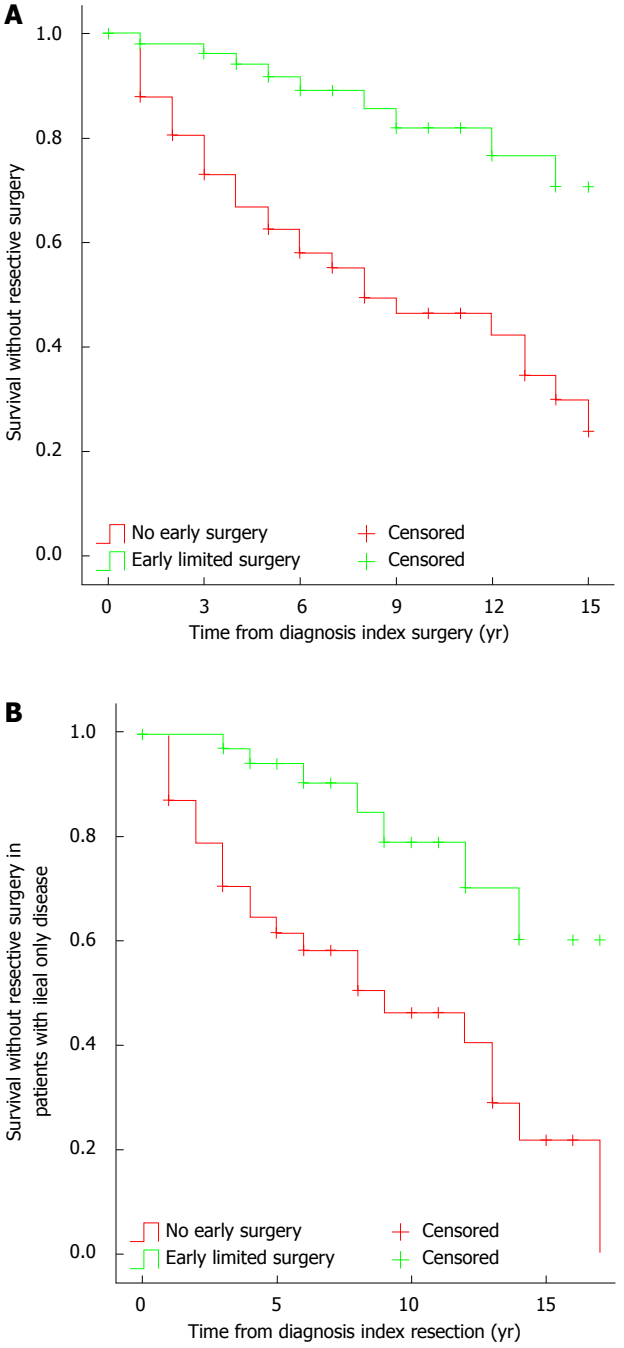


Figure 1 Need for surgery in Crohn's disease patients with and without an early limited surgery after matching on propensity scores. A: All Crohn's disease (CD) patients (n = 58), $P_{Logrank}$ < 0.001, HR = 0.23, 95%CI: 0.11-0.48; B: CD patients with ileal only disease location (n = 38). $P_{Logrank}$ < 0.001, HR = 0.25, 95%CI: 0.11-0.58.

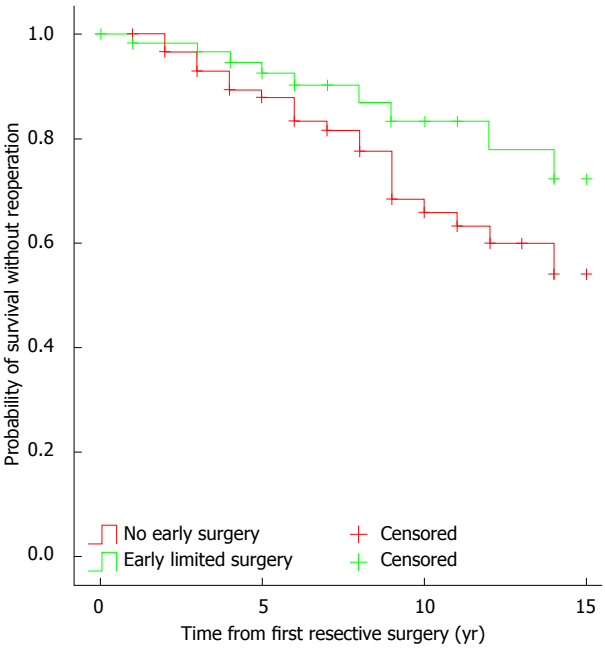


Figure 2 Need for reoperation in patients with and without early resective surgery ($P_{Logrank}$ = 0.038).

10 years ($P_{Logrank}$ < 0.001, HR = 0.25, 95%CI: 0.11-0.58) (Figure 1B).

Reoperation rates and predictors for reoperation

The need for reoperation in patients with early limited resection at 5 years was 7.5%, at 10 years it was 16.5%, while for those without early resective surgery at 5 years it was 12.9%, and 36.3% at 10 years in a Kaplan-Meier analysis ($P_{Logrank}$ = 0.038, Figure 2). In a Cox regression analysis, early limited surgery (P = 0.04) was the only factor independently associated with the need for reoperation (Table 5).

Reoperation rates after matching on propensity scores

In addition, we developed a propensity score model to assess further the need for reoperation in patients with and without early resective surgery. After identifying predictors, multivariate logistic regression was used to estimate propensity scores of early limited resection for each individual. Goodness-of-fit was evaluated by the Hosmer-Lemeshow test and P values were non-significant (P = 0.812). In the early limited surgery model, propensity scores ranged from 0.10 to 0.83 (median: 0.45) in patients with early limited resection (n = 63), and from 0.02 to 0.69 (median: 0.30) in patients with non-early surgery (n = 126). Using a 5-to-1 greedy matching algorithm, we were able to match 54 out of 63 (85.7%) patients with early limited surgery to patients with non-early surgery. As expected, the prevalence of factors included in the propensity score model was well balanced across surgical groups (data not shown).

The observed reoperation rates did not differ between the two groups (early surgery: 1.9%, 5.9%, and 17.7%; *vs* non-early surgery: 2%, 6.7%, and 25.1%, after 1, 5 and 10 years, respectively, $P_{Logrank}$ > 0.05, Figure 3A). Similar results were found if the analysis was restricted to patients with disease limited to the ileum only (n = 33, early surgery: 0%, 5.8%, and 20%, *vs* non-early surgery: 0%, 7.7%, and 14.3%, after 1, 5 and 10 years, respectively, $P_{Logrank}$ > 0.05, Figure 3B).

Table 5 Factors associated with the need for reoperation in Cox regression analysis

	P value	HR	95%CI
Early surgery[1]			
Yes	0.04	0.42	0.19-0.95
No		Reference	
Age at diagnosis			
A1	0.75	-	-
A2		Reference	
Disease location		0.95	
L2	0.77	-	-
[illegible]	0.95	-	-
		Reference	
Disease behavior at diagnosis		0.56	
B2	0.30	-	-
B3	0.65	-	-
B1		Reference	
Smoking			
Yes	0.29	-	-
No		Reference	

[1]Early resective surgery: Within the year of diagnosis.

DISCUSSION

In the present study, we studied the benefits of early limited resective surgery in patients in a population-based Veszprem Province database. Results from this population-based inception cohort have shown that surgery rates and overall exposure to steroids and biological agents were lower in patients with early limited resective surgery. In contrast, although patients with early limited resective surgery needed less reoperation, by Kaplan-Meier analysis and multivariate Cox regression analysis, in the final propensity-score-matched model, this advantage was lost, and the probability of reoperation was similar in patients with early limited resection and non-early surgery. To the best of our knowledge, this is the first time that the association between early limited resective surgery and reoperation were studied using a propensity score model.

The rates of resective surgery vary significantly according to previous studies with a range from 25% to 61% in the first 5 years. An earlier review article by Wolters *et al*[1] reported that the probability of first resective surgery was as high as 38%-96% within the first 15 years of diagnosis. The overall recurrence and reoperation rates after first resective surgery is 50%-60% and 28%-45%, respectively, during the subsequent 15 years. More recently, in the IBSEN Study, the cumulative probability of surgery was 13.6%, 27.0% and 37.9%, at 1, 5 and 10 years after diagnosis, respectively, while the risk of reoperation was also lower (9%)[16].

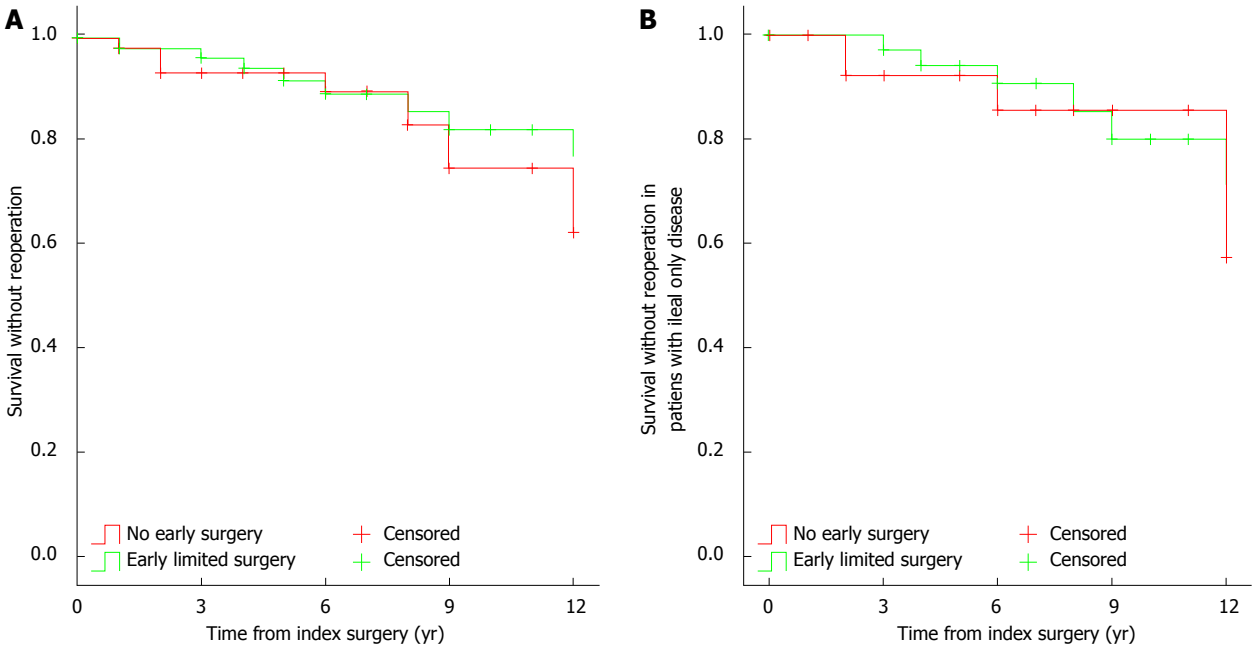


Figure 3 Need for reoperation in Crohn's disease patients with and without early resective surgery after matching on propensity scores. A: All Crohn's disease (CD) patients (n = 54). $P_{Logrank}$ > 0.05; B: CD patients with ileal only disease location (n = 33). $P_{Logrank}$ > 0.05.

Similarly, in a recent meta-analysis from the IOIBD Epidemiology Task Force, the authors reported that the probability of surgery in CD decreased gradually between 1955 and 2003, even before the advent of the biological era[3]. An association with increased and earlier use of immunosuppressants was also suggested in studies from Wales[6] and Hungary[5]. Ramadas *et al*[6] found that the rates of intestinal surgery decreased during the study period from 59% to 25% within 5 years of diagnosis. There was also a significant reduction in patients having any surgical procedure, from 60% to 35%. Likewise, in a previous referral center study from Hungary, early monotherapy with AZA or combination AZA/biological therapy was associated with a reduced risk for surgery. In earlier published studies from this cohort, the probability of surgical resection was 9.8%, 18.5% and 21.3% after 1, 3 and 5 years, respectively, in patients diagnosed between 2002 and 2006, and a recent decrease in surgical rates (in patients diagnosed after 1998) was observed[12]. These changes were associated with increased and earlier use of immunosuppressants[5]. Notwithstanding, the change in the use of immunosuppressants could be regarded as a marker of the complex changes in patient management rather than an exclusive factor itself.

Additional predictors of surgery include ileal or colonic disease location, complicated disease behavior, and age at onset - as reported previously in Sweden[17] and more recently from the IBSEN cohort[16]. Similarly, ileal (HR = 2.35) or ileocolonic (HR = 1.79) location compared to isolated colonic disease, as well as stricturing (HR = 4.33) or penetrating (HR = 3.44) disease at diagnosis, but not perianal disease, were independently associated with time to first surgery in a population-based cohort study from our research group[18]. Interestingly, we observed a similar phenotype pattern associated with early limited resection (ileal location, $P < 0.001$; complicated behavior, $P < 0.001$; and age at onset, $P = 0.06$).

However, the risk of surgery, as well as the disease course in patients with primary ileocecal CD, is somewhat different. In an earlier Swedish study, the risk of resective surgery in patients with primary ileocecal CD was 61%, 77% and 83%, after 1, 5 and 10 years of diagnosis, respectively, in 907 patients[19]. Relapse rates were 28% and 36% within 5 and 10 years of the first resection, respectively. In an Italian study, clinical and surgical recurrence rates after 5 years were 30.6% and 49.4%, and after 10 years they were and 3.6% and 28%, respectively[20]. In addition, early surgery (within 3 years of diagnosis) was associated with a longer postoperative course free from clinical recurrence compared with late surgery, but not with reoperation. In the present study, despite higher rates of ileal and complicated disease in patients with early limited surgery, the overall need of steroids (OR = 0.45, $P < 0.001$) during follow-up was lower, and there was a similar tendency for steroid-dependent disease course ($P = 0.07$) and need for biological agents ($P = 0.09$). In contrast, the overall use of AZA was similar in both groups, which may represent an active therapeutic decision by the treating physician rather than a marker of negative disease outcome. Of note, median follow-up was similar for both groups (12 years). We defined early surgery as that performed within 1 year of diagnosis, to avoid a potential bias due to early medical therapy. Of note, a positive effect of early aggressive medical therapy was already observed in patients in whom AZA was started within 18 mo of diagnosis, in a previous study in this cohort[5]. In addition, since reoperation rates may be lower in patients with extensive initial surgery due to the anatomical situa-

tion, we excluded these patients from the final analysis.

Comparable data were reported by Aratari *et al*[9]. In that study, early limited surgery at diagnosis was associated with less clinical recurrence - defined as need for steroids and lesions documented by endoscopy or radiology ($P = 0.01$), and less need for immunosuppressants ($P = 0.05$), but reoperation rates were not significantly different. Immunosuppressants were started in only 16.2% of patients with early or late surgery, confirming that the medical approach reported by the authors was much more conservative compared to the present study. In addition, the need for immunosuppressants was interpreted as a negative outcome. In another Italian study, the disease course of CD patients diagnosed during emergency surgery was compared to patients without emergency surgery[21]. The authors reported that the disease course was more benign in patients requiring surgery at diagnosis, by both univariate and multivariate analysis. Similar to the present study, surgery rates were significantly lower in patients operated on at diagnosis (in the present study with early limited surgery) compared to patients without surgery at diagnosis (in the present study without early limited surgery after matching on propensity scores). Observed surgery rates in the Italian study were 14% and 30% in the surgery-at-diagnosis group and 30% and 44% in the non-early surgery group, respectively. Of note, this design mimics clinical trials comparing two treatment algorithms: (1) early limited resective surgery; and (2) medical therapy but no early surgery. Observed surgery and drug exposure rates were clearly different. However, a different interpretation of our results may be that 40%-45% of patients could avoid surgery despite a similar patient phenotype within 10 years of diagnosis, while in the other group, all patients started with a limited operation. In addition, in the non-early surgery group, only 25.6% of patients received early AZA therapy. Thus, their therapeutic strategy was far from optimized. Moreover, reoperation rates were also analyzed in the present study, which would be very difficult in a clinical trial due to the need for long term follow-up. These were lower in patients with early limited resection versus those without early limited resection by Kaplan-Meier ($P_{Logrank} = 0.038$) and Cox regression ($P = 0.04$) analysis but the difference was lost after matching on propensity scores. Cost-benefit was not analyzed but with the increasing exposure to biological agents in certain CD populations[22], these studies are urgently awaited.

The authors are aware of the possible limitations of the present study. One such potential limitation is the partially retrospective nature of the study, which may have led to bias in data interpretation. However, data were collected prospectively since 1985 and intestinal resection can be considered an unbiased and solid criterion, even retrospectively, because the indications for surgical intervention are well established. Another possible criticism may be the definition of early surgery. In this subgroup, patients presented partly with a short history of subacute or even acute symptoms. In these patients, the timing of surgery was determined by the clinical presentation and not by the physician's strategic decision. However, this was also the case for a proportion of non-early surgical patients. Patient management has also changed significantly with regard to surgery techniques (laparoscopy) and imaging (availability of computed tomography and magnetic resonance imaging) that could have potentially affected therapeutic decision making, including indication for surgery. However, in the present study, one leading surgeon performed the majority of the operations and laparoscopic surgery and stricturoplasty was performed only in a minority of the cases. Similarly, there was only limited use of defunctioning ileostomy formation in routine management of CD during the follow-up period. Finally, postoperative management has significantly changed during the follow-up period, including routine endoscopy evaluation and prophylactic therapy; although in our analysis, we matched the groups for decade of diagnosis. In addition, there is accumulating evidence that anti-TNF therapies may reduce the number of operations[7,23], although this was not a universal finding[24,25]. Exposure to biological agents was limited in the present study, and most of these patients received induction-only or intermittent infliximab therapy. In contrast, the strengths of this study include the long-term, comprehensive, validated data capture, and the use of both propensity score matching and multiple Cox regression analysis to overcome the limitations present in any partly retrospective study, thereby enabling unbiased analysis. Moreover, a follow-up of several years is needed to assess the reoperation rates, especially in patients without early limited resection, which is almost impossible in a clinical trial.

In conclusion, early limited resective surgery was associated with a lower risk for surgery and lower overall exposure to steroids and biological agents in this population-based cohort but it was not preventive for reoperations after matching on propensity scores. In addition, the similar exposure to immunosuppressants in the two groups may be interpreted as an active medical decision rather than a negative disease outcome. Conversely, resective surgery could be avoided in 40%-45% of CD patients with a similar disease phenotype without an early limited surgery within 10 years of diagnosis.

COMMENTS

Background

The optimal initial therapy in patients with limited, isolated, stenotic ileocecal Crohn's disease (CD) is debated. In some cases, early surgery may represent a valid alternative to medical therapy.

Research frontiers

There are only limited data available on the disease course, including drug exposures, operation and reoperation rates in patients with and without an early limited resective surgery. In addition, data are lacking from population-based cohorts.

Innovations and breakthroughs

The present long-term, population-based study in a well-characterized cohort of patients with CD has found that the overall exposure to steroids and biologicals was lower in patients with early limited resective surgery, observed surgery

rates were also lower, yet reoperation rates were not different in the two groups after matching on propensity scores

Applications

Understanding the disease course in this subgroup of patients with CD may lead to more optimized patient managment and follow-up.

Terminology

Disease phenotype is categorized according to the Montreal classification and includes age at onset location and. Early limited surgery was defined as a resective surgery within the year of diagnosis and affecting only the terminal ileum and coecum.

Peer review

This is good research. To add impact, adding in other centres in Europe should be considered in future.

REFERENCES

1 **Wolters FL**, Russel MG, Stockbrügger RW. Systematic review: has disease outcome in Crohn's disease changed during the last four decades? *Aliment Pharmacol Ther* 2004; **20**: 483-496 [PMID: 15339320 DOI: 10.1111/j.1365-2036.2004.02123.x]

2 **Peyrin-Biroulet L**, Loftus EV, Colombel JF, Sandborn WJ. The natural history of adult Crohn's disease in population-based cohorts. *Am J Gastroenterol* 2010; **105**: 289-297 [PMID: 19861953 DOI: 10.1038/ajg.2009.579]

3 **Bernstein CN**, Loftus EV, Ng SC, Lakatos PL, Moum B. Hospitalisations and surgery in Crohn's disease. *Gut* 2012; **61**: 622-629 [PMID: 22267595 DOI: 10.1136/gutjnl-2011-301397]

4 **Jess T**, Riis L, Vind I, Winther KV, Borg S, Binder V, Langholz E, Thomsen OØ, Munkholm P. Changes in clinical characteristics, course, and prognosis of inflammatory bowel disease during the last 5 decades: a population-based study from Copenhagen, Denmark. *Inflamm Bowel Dis* 2007; **13**: 481-489 [PMID: 17206705 DOI: 10.1002/ibd.20036]

5 **Lakatos PL**, Golovics PA, David G, Pandur T, Erdelyi Z, Horvath A, Mester G, Balogh M, Szipocs I, Molnar C, Komaromi E, Veres G, Lovasz BD, Szathmari M, Kiss LS, Lakatos L. Has there been a change in the natural history of Crohn's disease? Surgical rates and medical management in a population-based inception cohort from Western Hungary between 1977-2009. *Am J Gastroenterol* 2012; **107**: 579-588 [PMID: 22233693 DOI: 10.1038/ajg.2011.448]

6 **Ramadas AV**, Gunesh S, Thomas GA, Williams GT, Hawthorne AB. Natural history of Crohn's disease in a population-based cohort from Cardiff (1986-2003): a study of changes in medical treatment and surgical resection rates. *Gut* 2010; **59**: 1200-1206 [PMID: 20650924 DOI: 10.1136/gut.2009.202101]

7 **Peyrin-Biroulet L**, Oussalah A, Williet N, Pillot C, Bresler L, Bigard MA. Impact of azathioprine and tumour necrosis factor antagonists on the need for surgery in newly diagnosed Crohn's disease. *Gut* 2011; **60**: 930-936 [PMID: 21228429 DOI: 10.1136/gut.2010.227884]

8 **Cosnes J**, Nion-Larmurier I, Beaugerie L, Afchain P, Tiret E, Gendre JP. Impact of the increasing use of immunosuppressants in Crohn's disease on the need for intestinal surgery. *Gut* 2005; **54**: 237-241 [PMID: 15647188 DOI: 10.1136/gut.2004.045294]

9 **Aratari A**, Papi C, Leandro G, Viscido A, Capurso L, Caprilli R. Early versus late surgery for ileo-caecal Crohn's disease. *Aliment Pharmacol Ther* 2007; **26**: 1303-1312 [PMID: 17848181 DOI: 10.1111/j.1365-2036.2007.03515.x]

10 **Lakatos L**, Mester G, Erdelyi Z, Balogh M, Szipocs I, Kamaras G, Lakatos PL. Striking elevation in incidence and prevalence of inflammatory bowel disease in a province of western Hungary between 1977-2001. *World J Gastroenterol* 2004; **10**: 404-409 [PMID: 14760767]

11 **Lakatos L**, Pandur T, David G, Balogh Z, Kuronya P, Tollas A, Lakatos PL. Association of extraintestinal manifestations of inflammatory bowel disease in a province of western Hungary with disease phenotype: results of a 25-year follow-up study. *World J Gastroenterol* 2003; **9**: 2300-2307 [PMID: 14562397]

12 **Lakatos L**, Kiss LS, David G, Pandur T, Erdelyi Z, Mester G, Balogh M, Szipocs I, Molnar C, Komaromi E, Lakatos PL. Incidence, disease phenotype at diagnosis, and early disease course in inflammatory bowel diseases in Western Hungary, 2002-2006. *Inflamm Bowel Dis* 2011; **17**: 2558-2565 [PMID: 22072315 DOI: 10.1002/ibd.21607]

13 **Silverberg MS**, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, Brant SR, Caprilli R, Colombel JF, Gasche C, Geboes K, Jewell DP, Karban A, Loftus EV, Peña AS, Riddell RH, Sachar DB, Schreiber S, Steinhart AH, Targan SR, Vermeire S, Warren BF. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can J Gastroenterol* 2005; **19** Suppl A: 5A-36A [PMID: 16151544]

14 **Stange EF**, Travis SP, Vermeire S, Beglinger C, Kupcinkas L, Geboes K, Barakauskiene A, Villanacci V, Von Herbay A, Warren BF, Gasche C, Tilg H, Schreiber SW, Schölmerich J, Reinisch W. European evidence based consensus on the diagnosis and management of Crohn's disease: definitions and diagnosis. *Gut* 2006; **55** Suppl 1: i1-15 [PMID: 16481628 DOI: 10.1136/gut.2005.081950a]

15 **Austin PC**. An Introduction to Propensity Score Methods for Reducing the Effects of Confounding in Observational Studies. *Multivariate Behav Res* 2011; **46**: 399-424 [PMID: 21818162 DOI: 10.1080/00273171.2011.568786]

16 **Solberg IC**, Vatn MH, Høie O, Stray N, Sauar J, Jahnsen J, Moum B, Lygren I. Clinical course in Crohn's disease: results of a Norwegian population-based ten-year follow-up study. *Clin Gastroenterol Hepatol* 2007; **5**: 1430-1438 [PMID: 18054751 DOI: 10.1016/j.cgh.2007.09.002]

17 **Bernell O**, Lapidus A, Hellers G. Risk factors for surgery and postoperative recurrence in Crohn's disease. *Ann Surg* 2000; **231**: 38-45 [PMID: 10636100]

18 **Szamosi T**, Banai J, Lakatos L, Czegledi Z, David G, Zsigmond F, Pandur T, Erdelyi Z, Gemela O, Papp M, Papp J, Lakatos PL. Early azathioprine/biological therapy is associated with decreased risk for first surgery and delays time to surgery but not reoperation in both smokers and nonsmokers with Crohn's disease, while smoking decreases the risk of colectomy in ulcerative colitis. *Eur J Gastroenterol Hepatol* 2010; **22**: 872-879 [PMID: 19648821 DOI: 10.1097/MEG.0b013e32833036d9]

19 **Bernell O**, Lapidus A, Hellers G. Risk factors for surgery and recurrence in 907 patients with primary ileocaecal Crohn's disease. *Br J Surg* 2000; **87**: 1697-1701 [PMID: 11122187 DOI: 10.1046/j.1365-2168.2000.01589.x]

20 **Margagnoni G**, Aratari A, Mangone M, Moretti A, Spagnolo A, Fascì Spurio F, Luchetti R, Papi C. Natural history of ileo-caecal Crohn's disease after surgical resection. A long term study. *Minerva Gastroenterol Dietol* 2011; **57**: 335-344 [PMID: 22105722]

21 **Latella G**, Cocco A, Angelucci E, Viscido A, Bacci S, Necozione S, Caprilli R. Clinical course of Crohn's disease first diagnosed at surgery for acute abdomen. *Dig Liver Dis* 2009; **41**: 269-276 [PMID: 18955023 DOI: 10.1016/j.dld.2008.09.010]

22 **van der Valk ME**, Mangen MJ, Leenders M, Dijkstra G, van Bodegraven AA, Fidder HH, de Jong DJ, Pierik M, van der Woude CJ, Romberg-Camps MJ, Clemens CH, Jansen JM, Mahmmod N, van de Meeberg PC, van der Meulen-de Jong AE, Ponsioen CY, Bolwerk CJ, Vermeijden JR, Siersema PD, van Oijen MG, Oldenburg B; on behalf of the COIN study group and the Dutch Initiative on Crohn and Colitis. Healthcare costs of inflammatory bowel disease have shifted from hospitalisation and surgery towards anti-TNFα therapy: results from the COIN study. *Gut* 2012 Nov 7; Epub ahead of print [PMID: 23135759 DOI: 10.1136/gutjnl-2012-303376]

23 **Schnitzler F**, Fidder H, Ferrante M, Noman M, Arijs I, Van Assche G, Hoffman I, Van Steen K, Vermeire S, Rutgeerts P. Long-term outcome of treatment with infliximab in 614 patients with Crohn's disease: results from a single-centre cohort. *Gut* 2009; **58**: 492-500 [PMID: 18832518 DOI: 10.1136/gut.2008.155812]

24 **Lazarev M**, Ullman T, Schraut WH, Kip KE, Saul M, Regueiro M. Small bowel resection rates in Crohn's disease and the indication for surgery over time: experience from a large tertiary care center. *Inflamm Bowel Dis* 2010; **16**: 830-835 [PMID: 19798731 DOI: 10.1002/ibd.21118]

25 **Domènech E**, Zabana Y, Garcia-Planella E, López San Román A, Nos P, Ginard D, Gordillo J, Martínez-Silva F, Beltrán B, Mañosa M, Cabré E, Gassull MA. Clinical outcome of newly diagnosed Crohn's disease: a comparative, retrospective study before and after infliximab availability. *Aliment Pharmacol Ther* 2010; **31**: 233-239 [PMID: 19832727 DOI: 10.1111/j.1365-2036.2009.04170.x]

P- Reviewer: Nash GF **S- Editor:** Zhai HH **L- Editor:** Kerr C
E- Editor: Ma S

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v19.i43.7711

World J Gastroenterol 2013 November 21; 19(43): 7711-7718
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Dendritic cell co-stimulatory and co-inhibitory markers in chronic HCV: An Egyptian study

Hanan Fouad, Maissa Saeed El Raziky, Rasha Ahmed Abdel Aziz, Dina Sabry, Ghada Mahmoud Abdel Aziz, Manal Ewais, Ahmed Reda Sayed

Hanan Fouad, Dina Sabry, Medical Biochemistry Department, Faculty of Medicine, Cairo University, 11562 Cairo, Egypt
Maissa Saeed El Raziky, Rasha Ahmed Abdel Aziz, Gastroenterology and Tropical Department, Faculty of Medicine, Cairo University, 11562 Cairo, Egypt
Ghada Mahmoud Abdel Aziz, Manal Ewais, Ahmed Reda Sayed, Medical Biochemistry Department, Faculty of Medicine, Beni Sueif University, 19206 Kornish Al Nile, Bani Sweif, Egypt
Author contributions: Abdel Aziz RA, Sabry D, Abdel Aziz GM, Ewais M and Sayed AR contributed to conception and design of the study, acquisition of data, conducting laboratory analytical work, drafting and editing of the article and final revision and approval of the completed article; and Fouad H and El Raziky MS contributed to analysis and interpretation of data, collection of human biological materials, drafting and editing of the article, revising the article for intellectual content and final revision and approval of the completed article.
Correspondence to: Hanan Fouad, Professor of Medical Biochemistry Department, Faculty of Medicine, Cairo University, 11562 Cairo, Egypt. hanan.fouad@kasralainy.edu.eg
Telephone: +20-122-7474888 Fax: +20-2-23632297
Received: April 27, 2013 Revised: July 30, 2013
Accepted: August 4, 2013
Published online: November 21, 2013

Abstract

AIM: To assess co-stimulatory and co-inhibitory markers of dendritic cells (DCs) in hepatitis C virus (HCV) infected subjects with and without uremia.

METHODS: Three subject groups were included in the study: group 1 involved 50 control subjects, group 2 involved 50 patients with chronic HCV infection and group 3 involved 50 HCV uremic subjects undergoing hemodialysis. CD83, CD86 and CD40 as co-stimulatory markers and PD-L1 as a co-inhibitory marker were assessed in peripheral blood mononuclear cells by real-time polymerase chain reaction. Interleukin-10 (IL-10) and hyaluronic acid (HA) levels were also assessed. All findings were correlated with disease activity, viral load and fibrogenesis.

RESULTS: There was a significant decrease in co-stimulatory markers; CD83, CD86 and CD40 in groups 2 and 3 *vs* the control group. Co-stimulatory markers were significantly higher in group 3 *vs* group 2. There was a significant elevation in PD-L1 in both HCV groups *vs* the control group. PD-L1 was significantly lower in group 3 *vs* group 2. There was a significant elevation in IL-10 and HA levels in groups 2 and 3, where IL-10 was higher in group 3 and HA was lower in group 3 *vs* group 2. HA level was significantly correlated with disease activity and fibrosis grade in group 2. IL-10 was significantly correlated with fibrosis grade in group 2. There were significant negative correlations between co-stimulatory markers and viral load in groups 2 and 3, except CD83 in dialysis patients. There was a significant positive correlation between PD-L1 and viral load in both HCV groups.

CONCLUSION: A significant decrease in DC co-stimulatory markers and a significant increase in a DC co-inhibitory marker were observed in HCV subjects and to a lesser extent in dialysis patients.

Key words: Hepatitis C virus; Uremia; Hemodialysis; Dendritic cells; CD83; CD86, CD40; PD-L1; Interleukin-10; Hyaluronic acid

Core tip: An assessment of the gene expression of co-stimulatory and a co-inhibitory marker (CD83, CD86, CD40, PD-L1) was conducted in patients with hepatitis C virus (HCV) infection and their correlations with viral load, hepatitis activity score and fibrosis grade were determined. There was a significant decrease in dendritic cell (DC) co-stimulatory markers in HCV infected

subjects, where HCV uremic subjects exhibited a lower degree of reduced co-stimulatory markers. There was a significant increase in the DC co-inhibitory marker in HCV infected subjects, where HCV uremic subjects exhibited a lower degree of increased co-inhibitory marker. All DC markers were significantly correlated with HCV viral load, hepatitis activity index and fibrosis score.

Fouad H, El Raziky MS, Abdel Aziz RA, Sabry D, Abdel Aziz GM, Ewais M, Sayed AR. Dendritic cell co-stimulatory and co-inhibitory markers in chronic HCV: An Egyptian study. *World J Gastroenterol* 2013; 19(43): 7711-7718 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7711.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7711

INTRODUCTION

Hepatitis C virus (HCV) infection is a major public health problem, with an estimated global prevalence of 3% occurring in about 180 million carriers and approximately 4 million people are newly infected annually[1]. It is estimated that up to 70% of individuals exposed to HCV develop viral persistence[2,3]. On average, over half a million people in Egypt are infected by HCV annually, far more than any other country in the world, according to a new study published in 2010[4].

A high prevalence of HCV has been reported among hemodialysis (HD) patients worldwide. The prevalence of HCV infection among HD patients is significantly higher than healthy blood donors and the general population[5]. HD patients may be at risk for HCV due to the involvement of multiple routes of infection, especially poor blood screening of transfused blood, low standard of dialysis procedures and the need to apply infection control practices.

Patients who spontaneously clear HCV infection have strong and broad T cell responses, while patients with chronic HCV have weak and functionally impaired responses characterized by poor proliferation, impaired cytotoxicity and reduced cytokine secretion after antigen exposure[6,7]. Dendritic cells (DCs) are efficient and potent antigen presenters and activators of antigen-specific T cells and adaptive immunity[8]. Defective DC activation of T cells may underlie poor T cell responsiveness in HCV infection, and may, in part, determine the response to therapy[9,10].

Human peripheral blood DCs are currently categorized into two major subsets: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). mDCs are effective antigen presenters to T cells and secrete interleukin 12, while pDCs are the most potent secretors of antiviral type-I interferons such as interferon α (IFN-α)[11]. DCs migrate to sites of inflammation, sample antigens, and integrate generic microbial danger signals *via* innate immune receptors, named pathogen recognition receptors (PRRs) that recognize pathogen-associated molecular patterns[12]. Signals from PRRs combine with signals from inflammatory cytokines to activate DCs, causing up-regulation of co-stimulatory molecules such as CD40 and CD86. DCs then migrate to lymphoid tissue where they activate antigen-specific CD4 and CD8 T cells by presenting antigens on major histocompatibility complex (MHC) class Ⅰ and Ⅱ molecules[13,14].

Reports of global immune dysfunction in HCV infection are controversial; some authors have found faulty responses to general PRRs stimulation including decreased IFNα and IL12 secretion, reduced CD86 expression, decreased HLA-DR (MHC class Ⅱ) and impaired stimulation of T cells in mixed lymphocyte reaction compared with normal controls[13]. Specific HCV proteins such as core and E2 can cause DC dysfunction in tissue culture models[14]. Other authors, including those using direct *ex vivo* human samples or a chimpanzee model of HCV have found no defects[15,16]. It has been consistently shown in HCV infection that pDC and mDC numbers are reduced in the peripheral compartment compared with normal controls, whereas reports have described increased numbers of DCs in the livers of HCV patients, suggesting hepatic DC sequestration[17-20]. The unresolved controversies listed above highlight the need for further study of DCs in HCV infection.

With regard to HD patients with HCV, some researchers reported altered monocyte-derived DC function in patients on HD[21]. However, reports on the natural history of hepatitis C in HD patients vary. Several studies stated that HCV disease activity in HD patients is mild, and is not progressive, perhaps due to immunological abnormalities in these patients[22].

The present study was conducted to assess DC response to HCV infection *via* assessment of the gene expression of co-stimulatory markers (CD83, CD86, and CD40) and a co-inhibitory marker (PD-L1) in pDCs and mDCs, and to study the correlations between DC functions and viral load, hepatitis activity score and fibrosis grade.

MATERIALS AND METHODS

The present study was conducted in the Hepatic Virology Center, Kasr Al-Ainy, Faculty of Medicine, Cairo University. The study involved Group I which included 50 healthy subjects of both genders aged 18-40 years representing the control group and 100 adult age- and sex-matched patients with HCV-related chronic liver disease (CLD).

The patients selected had to comply with the following inclusion criteria: HCV antibody-positive serum and HCV RNA-positive serum by reverse transcription polymerase chain reaction (RT/PCR) for more than 6 months. All patients had to comply with the following exclusion criteria: coinfection with HBV and HCV, hepatocellular carcinoma, severe psychiatric disease, serious co-morbid conditions, HIV-positive patients defined as

having a positive reaction to anti-HIV-1/2 (EIA), autoimmune hepatitis (positive reaction to antinuclear, anti-smooth muscle, anti-mitochondrial and anti-liver-kidney microsomal antibodies), schistosomiasis mansoni (patients with no previous history and negative stool examination), no previous history of regular use of hepatotoxic drugs or alcohol abuse (> 40 g of alcohol/d).

HCV patients were categorized into two groups: Group 2 included 50 HCV subjects with related CLD who were candidates for interferon therapy, and Group 3 included 50 HCV uremic subjects undergoing HD.

Patients were subjected to full clinical examination and abdominal ultrasonography. The following parameters were assessed in all subjects: serum levels of IL-10 and hyaluronic acid (HA) to assess fibrosis, as these parameters have been shown to be accurate in predicting significant fibrosis, severe fibrosis, and cirrhosis with area under characteristic curves (AUCs) of 0.73, 0.77 and 0.97, respectively. Moreover, accurate HA level cut-offs were defined for predicting significant fibrosis, severe fibrosis, and cirrhosis[23]. In addition, HA was an accurate noninvasive marker in predicting significant fibrosis in patients with hepatitis C on HD.

Quantitative gene expression of CD83, CD86, CD40 and PD-L1 in peripheral blood mononuclear cells was assessed by real-time PCR[24-27]. Histopathological examination of liver biopsy was performed using the Metavir scoring system for grading inflammation and staging fibrosis[28].

Whole blood samples were collected from all subjects. Serum was separated for assessment of HA and IL-10 levels by ELISA kits supplied by Corgenix Inc. (Westminster, CO, United States) according to the manufacturer's recommendations.

The peripheral blood mononuclear cell layer (buffy coat) was isolated using Histopaque-1077 (Sigma, St. Louis, MO, United States) and centrifuged at 400 *g* for 30 min. Total RNA was isolated from the buffy coat using RNeasy purification reagent (Qiagen, Valencia, CA, United States). cDNA was generated from 5 μg of total RNA extracted with 1 μL (20 pmol) antisense primer and 0.8 μL superscript AMV reverse transcriptase for 60 min at 37 ℃. The relative abundance of mRNA species was assessed using the SYBR® Green method on an ABI prism 7700 sequence detector system (Applied Biosystems, Foster City, CA, United States). PCR primers were designed with Primer-BLAST Software[29], (http://www.ncbi.nlm.nih.gov/tools/primer-blast) for RNA sequences from GenBank (Table 1). All primer sets had a calculated annealing temperature of 60 ℃. Quantitative RT-PCR was performed in duplicate in a 25 μL reaction volume consisting of 2X SYBR Green PCR Master Mix (Applied Biosystems, United States), 900 nmol/L of each primer and 2-3 μL of cDNA. Amplification conditions were 2 min at 50 ℃, 10 min at 95 ℃ and 40 cycles of denaturation for 15 s and annealing/extension at 60 ℃ for 10 min. Data from real-time assays were calculated using the v1.7 Sequence Detection Software from PE Biosystems (Foster City, CA, United States). Relative gene expression of CD83, CD86, CD40 and PD-L1 mRNA was calculated using the comparative Ct method as previously described. All values were normalized to the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene and reported as fold change over background levels detected in the control group.

Table 1 Real-time polymerase chain reaction primers of CD83, CD86, CD40 and PD-L1

Homo sapiens CD40	5'-CCA AAA CGG GCC CTG CTC CA-3'
	5'-GAG CCT GGC CCC CTC CAA CA-3'
	Gene Bank accession number NM_000074.2
Homo sapiens CD86	5'-TAG GAG GTA CGG GGA GCT CGC AA-3'
	5'-TTG GCA TGG CAG GTC TGC AGT C-3'
	Gene Bank accession number 006889.3
Homo sapiens CD83	5'-CGA CGC CGG AGG TGA AGG TG-3'
	5'-TCC GGG TCC TGC AGA GTG CA-3'
	Gene Bank accession number 001040280.1
Homo sapiens PDL1	5'-ACA GAG GGC CCG GCT GTT GA-3'
	5'-CTT CGG CCT TGG GGT AGC CC-3'
	Gene Bank accession number AY254342.1
Homo sapiens GAPDH	5'-GAAGGTGAAGGTCGGAGTCA-3'
	5'-GAAGATGGTGATGGGATTTC-3'
	Gene Bank accession number NC_000019.9

PDL1: Programmed death ligand 1; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis

The SPSS program version 16.0.1 (SPSS Inc., Chicago, IL, United States) was used. Numerical data were expressed as mean ± SD. For comparisons between treatment groups, the null hypothesis was tested by a single-factor ANOVA for multiple groups or unpaired *t* test for two groups. Comparisons were considered statistically significant if $P < 0.05$. Spearman correlations were assessed between certain studied parameters.

RESULTS

The results showed that there was a significant decrease in all co-stimulatory markers; CD83, CD86 and CD40 in group 2 (HCV subjects) and in group 3 (HCV subjects on HD) in comparison to control subjects. Co-stimulatory markers were significantly higher in group 3 in comparison to group 2. With regard to PD-L1, there was a significant increase in groups 2 and 3 in comparison to control subjects. PD-L1 was significantly lower in group 3 as compared to group 2 (Figure 1). This was reflected in viral load, where significant negative correlations were observed between all co-stimulatory markers and viral load in groups 2 and 3, except CD83 in dialysis patients (Figure 2)

The findings in the present study showed a significant elevation in IL-10 and in HA levels in groups 2 and 3, where IL-10 was more significantly elevated in group 3 and HA was significantly lower in group 3 in comparison to group 2 (Figure 3).

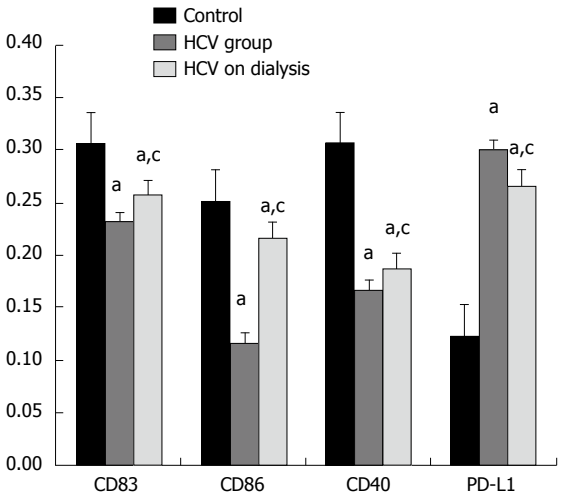


Figure 1 Relative quantitative gene expression of CD83, CD86, CD40 and PD-L1 in all study subjects expressed as mean ± SD. [a]*P* < 0.05 in both hepatitis C virus (HCV) patient groups *vs* control group; [c]*P* < 0.05 in the HCV patient group *vs* HCV uremic patients on hemodialysis.

In group 2, the results showed that there was a significant correlation between HA and hepatitis activity score as well as grade of fibrosis; $P < 0.001$. No correlation between IL-10 levels and hepatitis activity score was observed, whereas, there was a significant positive correlation between IL-10 and fibrosis grade, $P < 0.001$ (Figure 4). Biopsy samples were not taken from group 3 patients (HCV on dialysis).

The results showed that there were significant negative correlations between all co-stimulatory markers and viral load in groups 2 and 3, except CD83 in dialysis patients. There was a significant positive correlation between PD-L1 and viral load in both HCV groups (Figure 2).

Of the 50 non-uremic patients who were candidates for interferon therapy only 4 remained PCR positive for HCV after treatment.

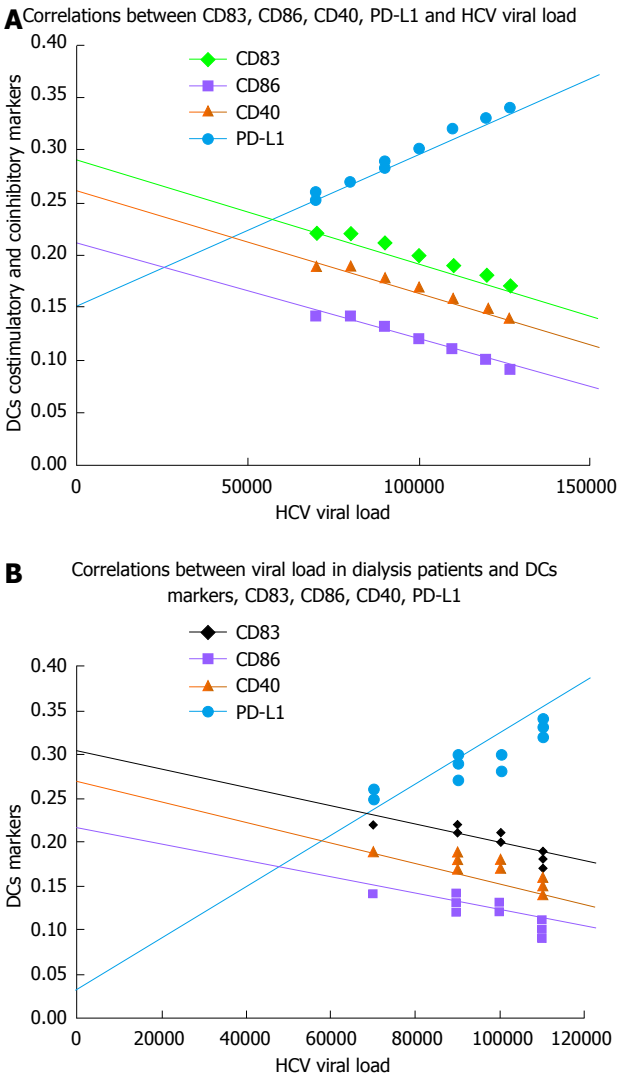


Figure 2 Correlations. A: Between hepatitis C viremia and CD83. $r = -0.98$, $P < 0.001$, CD86: $r = -0.866$, $P < 0.001$, CD40: $r = -0.98$, $P < 0.001$, PD-L1: $r = 0.889$, $P < 0.001$; B: Between hepatitis C viremia in uremic patients on hemodialysis and CD83. $r = -0.096$, $P > 0.05$ NS, CD86: $r = -0.588$, $P < 0.05$, CD40: $r = -0.946$, $P < 0.001$, PD-L1: $r = 0.663$, $P < 0.05$. HCV: Hepatitis C virus; DC: Dendritic cell

DISCUSSION

The results of the present study showed that in HCV infected subjects peripheral blood mononuclear cells exhibited lower expression of co-stimulatory markers; CD83, CD86, and CD40 and higher expression of a co-inhibitory marker; PD-L1 in comparison to healthy control subjects. Various studies have assessed DC function in HCV infection. Some have reported that DC function was impaired in HCV infection, which was identified by impaired allostimulatory capacity, decreased DC frequencies, increased mDC IL-10 secretion and decreased IL-12 secretion, as well as decreased pDC IFN-α secretion, while others did not[15,17,30].

Our findings coincide with the results reported by MacDonald *et al*[12], who stated that monocyte-derived DCs from patients with chronic HCV infection were significantly defective in their capacity to up-regulate the expression of surface molecules involved in antigen presentation (HLA-DR, CD86, CD40) and a classical marker of DC activation (CD83) and at physiological ratios of DCs to T cells, and decreased their ability to present antigen in allogeneic mixed lymphocyte reaction (MLR) assays. More recently, Shen *et al*[24] reported that impaired HCV-specific T cell immunity was associated with the persistence of HCV infection. DC dysfunction was believed to be involved in impaired T cell immunity, but the mechanisms are not understood. The results showed that the expression of both co-stimulatory markers (CD83, CD86, and CD40) and a co-inhibitory marker (PD-L1) was imbalanced in HCV-infected patients compared with healthy controls. The PD-L1/CD86 ratio was increased and positively correlated with PD-L1 expression on DCs in HCV-infected patients. The allostimulatory capacity of DCs was impaired and inversely correlated with PD-L1 expression and the PD-L1/CD86 ratio. These findings agree with our study and suggest that the effect of inhibitory marker PD-L1 overwhelmed the effect of co-

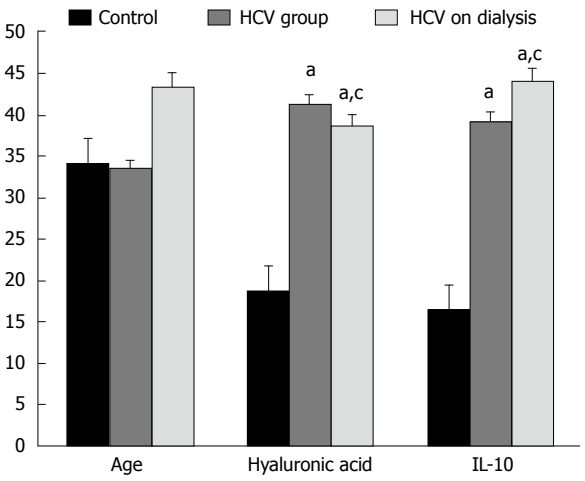


Figure 3 Serum levels of hyaluronic acid (ng/mL) and interleukin-10 (pg/mL) in all study subjects expressed as mean ± SD. [a]*P* < 0.05 *vs* control group, [c]*P* < 0.05 in the hepatitis C virus (HCV) patient group *vs* HCV uremic patients on hemodialysis. IL-10: Interleukin-10.

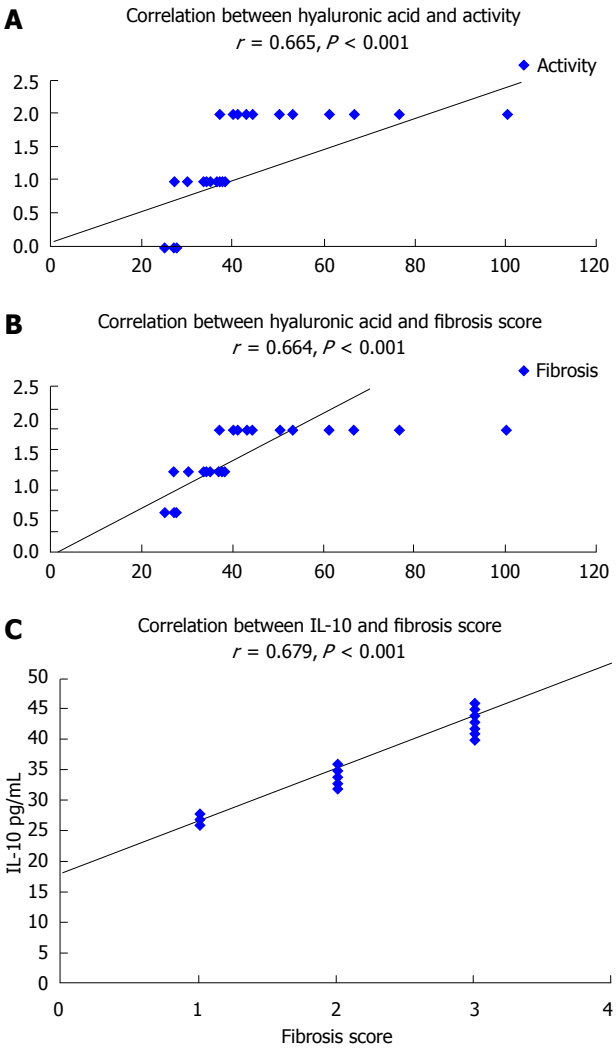


Figure 4 Correlation analysis. A: Between hyaluronic acid levels and hepatitis activity score (*P* < 0.001); B: Between hyaluronic acid levels and fibrosis score (*P* < 0.001); C: Between interleukin-10 (IL-10) levels and fibrosis score (*P* < 0.001).

stimulatory markers and down-regulated DC-T activation in HCV-infected patients.

HCV may inhibit the immune response of DCs, hindering the adaptive response from T cells[31]. It was hypothesized that DC dysfunction may determine the response to PEG-IFN/ribavirin therapy. In the study by Mengshol *et al*[32], the authors found that pDCs and mDCs were decreased compared to normal controls, consistent with prior studies in HCV and similar to prior studies of patients with HIV[33].

In the present study, the results of HA assessment revealed a significant elevation in HCV infected subjects in comparison to the healthy control group. Moreover, HA levels were positively correlated with hepatitis activity score and grade of fibrosis. These findings can be explained by a recent study conducted on another virus by Spahn *et al*[34], who stated that ineffective CD8 (+) T cell immunity to adeno-associated virus can result in prolonged liver injury and fibrogenesis. More recently, Jiao *et al*[35] determined whether liver DCs play a role in enhancing regression of liver fibrosis in murine carbon tetrachloride-induced liver injury. They found that conditional DC depletion soon after discontinuation of the liver insult led to delayed regression of fibrosis and reduced clearance of activated hepatic stellate cells, the key fibrogenic cells in the liver. Conversely, DC expansion induced either by Flt3L (fms-like tyrosine kinase-3 ligand) or adoptive transfer of purified DCs accelerated liver fibrosis regression. DC modulation of fibrosis was partially dependent on matrix metalloproteinase (MMP)-9, as MMP-9 inhibition abolished the Flt3L-mediated effect and the ability of transferred DCs to accelerate regression of fibrosis. In contrast, transfer of DCs from MMP-9-deficient mice failed to improve fibrosis regression. Another study conducted by Ryan *et al*[36] proved that in HCV chronically infected subjects, a single nucleotide polymorphism in a c-type lectin expressed by DCs, CD209, was associated with more advanced liver disease and with significantly higher liver fibrosis scores.

With regard to IL-10 levels in our study, the findings revealed significantly elevated IL-10 levels in HCV infected subjects in comparison to healthy controls. Moreover, IL-10 levels were positively correlated with fibrosis grade. These findings agree with results reported by Díaz-Valdés *et al*[37], who stated that high levels of IL-10 present in chronic HCV infection are associated with the poor antiviral cellular immune responses found in these patients. To overcome the immunosuppressive effect of IL-10 on antigen-presenting cells such as DCs, they developed peptide inhibitors of IL-10 and found that IL-10 inhibiting peptides have important applications in enhancing anti-HCV immune responses by restoring the immunostimulatory capabilities of DCs.

With regard to HD patients, Choi *et al*[21] found that surface expression of major histocompatibility complex class Ⅱ, CD83, and CD86, and chemokine receptor CCR7 in monocyte derived dendritic cells (moDCs) was

not different between HD patients and healthy controls. Furthermore, moDCs from HD patients produced significantly higher amounts of IL-6, IL-8, IL-1b, and TNF-α when stimulated by cytokine cocktails compared to healthy controls. Abnormalities in cytokine production by moDCs in ESRD patients have also been reported in several previous studies[38,39]. Verkade *et al*[39] also demonstrated a marked increase in IL-15 production, a known stimulatory cytokine for DCs, while IL-10 and IL-12p70 levels were not different. In addition, mature moDCs from HD patients showed significantly enhanced allogeneic T cell proliferation compared to healthy controls. These findings could explain our results which showed a significantly lower degree of reduced co-stimulatory markers as well as a significantly lower degree of elevated co-inhibitory marker in HD patients as compared to HCV subjects without uremia.

In conclusion, a significant decrease in DC co-stimulatory markers in HCV infected subjects was observed, where HCV uremic subjects exhibited a lower degree of reduced co-stimulatory markers. There was a significant increase in the DC co-inhibitory marker in HCV infected subjects, where HCV uremic subjects exhibited a lower degree of elevated co-inhibitory marker. All DC markers were significantly correlated with HCV viral load, hepatitis activity index and fibrosis score.

COMMENTS

Background

Defective dendritic cell (DC) activation of T cells may underlie poor T cell responsiveness in hepatitis C virus (HCV) infection, and may, in part, determine the response to therapy. It has been consistently shown in HCV infection that plasmacytoid DCs (pDCs) and myeloid DCs (mDCs) numbers are reduced in the peripheral compartment compared with normal controls. Other reports have described increased numbers of DCs in the livers of HCV patients, suggesting hepatic DC sequestration. The unresolved controversies listed above highlight the need for further study of DCs in HCV infection. With regard to hemodialysis (HD) patients with HCV, some researchers have reported altered monocyte-derived dendritic cell function in patients on HD. However, reports on the natural history of hepatitis C in HD patients vary. Several studies stated that HCV disease activity in HD patients is mild, and is not progressive, perhaps due to immunological abnormalities in these patients. The present study was conducted to assess DC response to HCV infection with and without uremia *via* assessment of the gene expression of co-stimulatory markers (CD83, CD86, and CD40) and a co-inhibitory marker (PD-L1) in pDCs and mDCs, and to study the correlations between DC functions and viral load, hepatitis activity score and fibrosis grade.

Research frontiers

Researchers have recently explored the mechanisms by which DC function is regulated during HCV infection, leading to impaired antiviral T cell responses and so to persistent viral infection. Recently, DC-based vaccines against HCV have been developed. Several studies describe the current understanding of DC function during HCV infection and explore the prospects of DC-based HCV vaccines. In particular, they describe the biology of DC, the phenotype of DC in HCV-infected patients, the effect of HCV on DC development and function, studies on new DC-based vaccines against HCV infection, and strategies to improve the efficacy of DC-based vaccines.

Innovations and breakthroughs

A recent study stated that the immature pDC phenotype and sustained pDC and mDC hyperresponsiveness are associated with spontaneous resolution of acute HCV infection. Several investigators found that injection of DCs presenting viral proteins constitutes a promising approach to stimulate T cell immunity against HCV. They also describe the strategy implemented to enhance antigen loading and immunostimulatory functions of DCs used in the preparation of therapeutic vaccines.

Applications

Assessment of DC functions in HCV patients may be applicable to anticipate the response to HCV standard of care therapy and to assign patients to specific therapeutic protocols. DC-based immunotherapy may be used in selected HCV cases with poor therapeutic response, high fibrosis index and high hepatitis activity score.

Terminology

Plasmacytoid DCs (pDCs), myeloid DCs (mDCs), interferon α (IFNα), pathogen recognition receptors (PRRs), pathogen-associated molecular patterns (PAMPs), co-stimulatory markers (CD83, CD86, and CD40), a co-inhibitory marker (PD-L1), matrix metalloproteinase (MMP).

Peer review

Interesting subject with sufficiently large cohorts to detect difference in response to Hepatitis C infection in those with liver disease *vs* others under dialysis treatment for chronic kidney failure. The key finding that there is "a significant decrease in dendritic cell co-stimulatory markers in HCV infected subjects, where HCV uremic subjects exhibited lower degree of the decrease. There was a significant increase in dendritic cell co-inhibitory marker in HCV infected subjects, where HCV uremic subjects exhibited lower degree of the elevation." If proved in an individual HCV patient may prove important in directing management of HCV infected persons.

REFERENCES

1 **Khan S**, Attaullah S, Ali I, Ayaz S, Naseemullah SN, Siraj S, Khan J. Rising burden of Hepatitis C Virus in hemodialysis patients. *Virol J* 2011; **8**: 438 [PMID: 21920054 DOI: 10.1186/1743-422X-8-438]

2 **Guerra J**, Garenne M, Mohamed MK, Fontanet A. HCV burden of infection in Egypt: results from a nationwide survey. *J Viral Hepat* 2012; **19**: 560-567 [PMID: 22762140 DOI: 10.1111/j.1365-2893.2011.01576.x]

3 **Sievert W**, Altraif I, Razavi HA, Abdo A, Ahmed EA, Alomair A, Amarapurkar D, Chen CH, Dou X, El Khayat H, Elshazly M, Esmat G, Guan R, Han KH, Koike K, Largen A, McCaughan G, Mogawer S, Monis A, Nawaz A, Piratvisuth T, Sanai FM, Sharara AI, Sibbel S, Sood A, Suh DJ, Wallace C, Young K, Negro F. A systematic review of hepatitis C virus epidemiology in Asia, Australia and Egypt. *Liver Int* 2011; **31** Suppl 2: 61-80 [PMID: 21651703 DOI: 10.1111/j.1478-3231.2011.02540.x]

4 **Miller FD**, Abu-Raddad LJ. Evidence of intense ongoing endemic transmission of hepatitis C virus in Egypt. *Proc Natl Acad Sci USA* 2010; **107**: 14757-14762 [PMID: 20696911 DOI: 10.1073/pnas.1008877107]

5 **Khodir SA**, Alghateb M, Okasha KM, Shalaby Sel-S. Prevalence of HCV infections among hemodialysis patients in Al Gharbiyah Governorate, Egypt. *Arab J Nephrol Transplant* 2012; **5**: 145-147 [PMID: 22967252]

6 **Centers for Disease Control and Prevention (CDC)**. Progress toward prevention and control of hepatitis C virus infection--Egypt, 2001-2012. *MMWR Morb Mortal Wkly Rep* 2012; **61**: 545-549 [PMID: 22832935]

7 **Bowen DG**, Walker CM. Adaptive immune responses in acute and chronic hepatitis C virus infection. *Nature* 2005; **436**: 946-952 [PMID: 16107834 DOI: 10.1038/nature04079]

8 **Pembroke T**, Rees I, Gallagher K, Jones E, Mizen P, Navruzov T, Freedman A, Fielding C, Humphreys IR, Wang EC, Gallimore AM, Godkin A. Rapid early innate control of hepatitis C virus during IFN-α treatment compromises adaptive CD4+ T-cell immunity. *Eur J Immunol* 2012; **42**: 2383-2394 [PMID: 22653709 DOI: 10.1002/eji.201142072]

9 **Gordon FD**, Kwo P, Ghalib R, Crippin J, Vargas HE, Brown KA, Schiano T, Chaudhri E, Pedicone LD, Brown RS. Peginterferon-α-2b and ribavirin for hepatitis C recurrence

postorthotopic liver transplantation. *J Clin Gastroenterol* 2012; **46**: 700-708 [PMID: 22739223]
10 **Gruener NH**, Lechner F, Jung MC, Diepolder H, Gerlach T, Lauer G, Walker B, Sullivan J, Phillips R, Pape GR, Klenerman P. Sustained dysfunction of antiviral CD8+ T lymphocytes after infection with hepatitis C virus. *J Virol* 2001; **75**: 5550-5558 [PMID: 11356962 DOI: 10.1128/JVI.75.12.5550-5558]
11 **Rehermann B**. Hepatitis C virus versus innate and adaptive immune responses: a tale of coevolution and coexistence. *J Clin Invest* 2009; **119**: 1745-1754 [PMID: 19587449 DOI: 10.1172/JCI39133]
12 **MacDonald AJ**, Semper AE, Libri NA, Rosenberg WM. Monocyte-derived dendritic cell function in chronic hepatitis C is impaired at physiological numbers of dendritic cells. *Clin Exp Immunol* 2007; **148**: 494-500 [PMID: 17362265 DOI: 10.1111/j.1365-2249.2007.03367.x]
13 **Dolganiuc A**, Chang S, Kodys K, Mandrekar P, Bakis G, Cormier M, Szabo G. Hepatitis C virus (HCV) core protein-induced, monocyte-mediated mechanisms of reduced IFN-alpha and plasmacytoid dendritic cell loss in chronic HCV infection. *J Immunol* 2006; **177**: 6758-6768 [PMID: 17082589]
14 **Kanto T**, Hayashi N, Takehara T, Tatsumi T, Kuzushita N, Ito A, Sasaki Y, Kasahara A, Hori M. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. *J Immunol* 1999; **162**: 5584-5591 [PMID: 10228041]
15 **Szabo G**, Chang S, Dolganiuc A. Altered innate immunity in chronic hepatitis C infection: cause or effect? *Hepatology* 2007; **46**: 1279-1290 [PMID: 17886338 DOI: 10.1002/hep.21938]
16 **Velazquez VM**, Hon H, Ibegbu C, Knechtle SJ, Kirk AD, Grakoui A. Hepatic enrichment and activation of myeloid dendritic cells during chronic hepatitis C virus infection. *Hepatology* 2012; **56**: 2071-2081 [PMID: 22711645 DOI: 10.1002/hep.25904]
17 **Martinet J**, Leroy V, Dufeu-Duchesne T, Larrat S, Richard MJ, Zoulim F, Plumas J, Aspord C. Plasmacytoid dendritic cells induce efficient stimulation of antiviral immunity in the context of chronic hepatitis B virus infection. *Hepatology* 2012; **56**: 1706-1718 [PMID: 22707082 DOI: 10.1002/hep.25879]
18 **Diebold SS**, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 2004; **303**: 1529-1531 [PMID: 14976261 DOI: 10.1126/science.1093616]
19 **Otsuka M**, Kato N, Moriyama M, Taniguchi H, Wang Y, Dharel N, Kawabe T, Omata M. Interaction between the HCV NS3 protein and the host TBK1 protein leads to inhibition of cellular antiviral responses. *Hepatology* 2005; **41**: 1004-1012 [PMID: 15841462 DOI: 10.1002/hep.20666]
20 **Sioud M**. Innate sensing of self and non-self RNAs by Toll-like receptors. *Trends Mol Med* 2006; **12**: 167-176 [PMID: 16530484 DOI: 10.1016/j.molmed.2006.02.004]
21 **Choi HM**, Woo YS, Kim MG, Jo SK, Cho WY, Kim HK. Altered monocyte-derived dendritic cell function in patients on hemodialysis: a culprit for underlying impaired immune responses. *Clin Exp Nephrol* 2011; **15**: 546-553 [PMID: 21360015 DOI: 10.1007/s10157-011-0424-2]
22 **Bahadi A**, Maoujoud O, Zejjari Y, Alayoud A, Hassani K, Elkabbaj D, Benyahia M. [Diagnosis and evaluation of hepatitis C virus among haemodialysis patients]. *East Mediterr Health J* 2013; **19**: 192-199 [PMID: 23516832]
23 **Avila RE**, Carmo RA, Farah Kde P, Teixeira AL, Coimbra LV, Antunes CM, Lambertucci JR. Hyaluronic acid in the evaluation of liver fibrosis in patients with hepatitis C on haemodialysis. *Braz J Infect Dis* 2010; **14**: 335-341 [PMID: 20963316 DOI: 10.1590/S1413-86702010000400004]
24 **Shen T**, Chen X, Chen Y, Xu Q, Lu F, Liu S. Increased PD-L1 expression and PD-L1/CD86 ratio on dendritic cells were associated with impaired dendritic cells function in HCV infection. *J Med Virol* 2010; **82**: 1152-1159 [PMID: 20513078 DOI: 10.1002/jmv.21809]
25 **Pachiadakis I**, Chokshi S, Cooksley H, Farmakiotis D, Sarrazin C, Zeuzem S, Michalak TI, Naoumov NV. Early viraemia clearance during antiviral therapy of chronic hepatitis C improves dendritic cell functions. *Clin Immunol* 2009; **131**: 415-425 [PMID: 19303818 DOI: 10.1016/j.clim.2009.02.001]
26 **Szabo G**, Dolganiuc A. Subversion of plasmacytoid and myeloid dendritic cell functions in chronic HCV infection. *Immunobiology* 2005; **210**: 237-247 [PMID: 16164031]
27 **Barnes E**, Salio M, Cerundolo V, Francesco L, Pardoll D, Klenerman P, Cox A. Monocyte derived dendritic cells retain their functional capacity in patients following infection with hepatitis C virus. *J Viral Hepat* 2008; **15**: 219-228 [PMID: 18194173 DOI: 10.1111/j.1365-2893.2007.00934.x]
28 **Theise ND**. Liver biopsy assessment in chronic viral hepatitis: a personal, practical approach. *Mod Pathol* 2007; **20** Suppl 1: S3-14 [PMID: 17486049 DOI: 10.1038/modpathol.3800693]
29 **Ye J**, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* 2012; **13**: 134 [PMID: 22708584 DOI: 10.1186/1471-2105-13-134]
30 **Kanto T**, Inoue M, Miyazaki M, Itose I, Miyatake H, Sakakibara M, Yakushijin T, Kaimori A, Oki C, Hiramatsu N, Kasahara A, Hayashi N. Impaired function of dendritic cells circulating in patients infected with hepatitis C virus who have persistently normal alanine aminotransferase levels. *Intervirology* 2006; **49**: 58-63 [PMID: 16166790 DOI: 10.1159/000087264)]
31 **Shiina M**, Rehermann B. Cell culture-produced hepatitis C virus impairs plasmacytoid dendritic cell function. *Hepatology* 2008; **47**: 385-395 [PMID: 18064579 DOI: 10.1002/hep.21996]
32 **Mengshol JA**, Golden-Mason L, Castelblanco N, Im KA, Dillon SM, Wilson CC, Rosen HR. Impaired plasmacytoid dendritic cell maturation and differential chemotaxis in chronic hepatitis C virus: associations with antiviral treatment outcomes. *Gut* 2009; **58**: 964-973 [PMID: 19193669 DOI: 10.1136/gut.2008.168948]
33 **Barron MA**, Blyveis N, Palmer BE, MaWhinney S, Wilson CC. Influence of plasma viremia on defects in number and immunophenotype of blood dendritic cell subsets in human immunodeficiency virus 1-infected individuals. *J Infect Dis* 2003; **187**: 26-37 [PMID: 12508143 DOI: 10.1086/345957]
34 **Spahn J**, Pierce RH, Crispe IN. Ineffective CD8(+) T-cell immunity to adeno-associated virus can result in prolonged liver injury and fibrogenesis. *Am J Pathol* 2011; **179**: 2370-2381 [PMID: 21925469 DOI: 10.1016/j.ajpath.2011.08.004]
35 **Jiao J**, Sastre D, Fiel MI, Lee UE, Ghiassi-Nejad Z, Ginhoux F, Vivier E, Friedman SL, Merad M, Aloman C. Dendritic cell regulation of carbon tetrachloride-induced murine liver fibrosis regression. *Hepatology* 2012; **55**: 244-255 [PMID: 21898476 DOI: 10.1002/hep.24621]
36 **Ryan EJ**, Dring M, Ryan CM, McNulty C, Stevenson NJ, Lawless MW, Crowe J, Nolan N, Hegarty JE, O'Farrelly C. Variant in CD209 promoter is associated with severity of liver disease in chronic hepatitis C virus infection. *Hum Immunol* 2010; **71**: 829-832 [PMID: 20470843 DOI: 10.1016/j.humimm.2010.05.007]
37 **Díaz-Valdés N**, Manterola L, Belsúe V, Riezu-Boj JI, Larrea E, Echeverria I, Llópiz D, López-Sagaseta J, Lerat H, Pawlotsky JM, Prieto J, Lasarte JJ, Borrás-Cuesta F, Sarobe P. Improved dendritic cell-based immunization against hepatitis C virus using peptide inhibitors of interleukin 10. *Hepatology* 2011; **53**: 23-31 [PMID: 21154952 DOI: 10.1002/hep.23980]
38 **Agrawal S**, Gollapudi P, Elahimehr R, Pahl MV, Vaziri ND. Effects of end-stage renal disease and haemodialysis on

dendritic cell subsets and basal and LPS-stimulated cytokine production. *Nephrol Dial Transplant* 2010; **25**: 737-746 [PMID: 19903659 DOI: 10.1093/ndt/gfp580]
39 **Verkade MA**, van Druningen CJ, Vaessen LM, Hesselink DA, Weimar W, Betjes MG. Functional impairment of monocyte-derived dendritic cells in patients with severe chronic kidney disease. *Nephrol Dial Transplant* 2007; **22**: 128-138 [PMID: 17005525 DOI: 10.1093/ndt/gfl519]

P- Reviewers: Madalinski KZ, Friedman EA **S- Editor:** Wen LL
L- Editor: Webster JR **E- Editor:** Ma S

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v19.i43.7719

World J Gastroenterol 2013 November 21; 19(43): 7719-7725
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Characteristics of nonvariceal upper gastrointestinal hemorrhage in patients with chronic kidney disease

Chang Seok Bang, Yong Seop Lee, Yun Hyeong Lee, Hotaik Sung, Hong Jun Park, Hyun Soo Kim, Jin Bong Kim, Gwang Ho Baik, Yeon Soo Kim, Jai Hoon Yoon, Dong Joon Kim, Ki Tae Suk

Chang Seok Bang, Yong Seop Lee, Yun Hyeong Lee, Jin Bong Kim, Gwang Ho Baik, Yeon Soo Kim, Jai Hoon Yoon, Dong Joon Kim, Ki Tae Suk, Department of Internal Medicine, Hallym University College of Medicine, Chuncheon 200-704, South Korea
Hotaik Sung, Department of Biology, Stanford University, Stanford, CA 94305, United States
Hong Jun Park, Hyun Soo Kim, Department of Internal Medicine, Yonsei University Wonju College of Medicine, Wonju 220-786, South Korea
Author contributions: Suk KT and Bang CS designed research; Bang CS, Lee YS, Lee YH, Kim JB, Baik GH, Kim YS and Yoon JH performed research; Sung H, Park HJ, Kim HS and Kim DJ contributed new reagent/analytic tools; Bang CS and Suk KT analyzed data; and Bang CS wrote the paper.
Supported by The Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2010-0021482) to Suk KT
Correspondence to: Ki Tae Suk, MD, PhD, Department of Internal Medicine, Hallym University College of Medicine, Kyodong, Chuncheon 200-704, South Korea. ktsuk@hallym.ac.kr
Telephone: +82-33-240-5526 Fax: +82-33-241-8064
Received: July 23, 2013 Revised: September 4, 2013
Accepted: September 15, 2013
Published online: November 21, 2013

Abstract

AIM: To evaluate the clinical characteristics of nonvariceal upper gastrointestinal hemorrhage (NGIH) in patients with chronic kidney disease (CKD).

METHODS: From 2003 to 2010, a total of 72 CKD patients (male *n* = 52, 72.2%; female *n* = 20, 27.8%) who had undergone endoscopic treatments for NGIH were retrospectively identified. Clinical findings, endoscopic features, prognosis, rebleeding risk factors, and mortality-related factors were evaluated. The characteristics of the patients and rebleeding-related data were recorded for the following variables: gender, age, alcohol use and smoking history, past hemorrhage history, endoscopic findings (the cause, location, and size of the hemorrhage and the hemorrhagic state), therapeutic options for endoscopy, endoscopist experience, clinical outcomes, and mortality.

RESULTS: The average size of the hemorrhagic site was 13.7 ± 10.2 mm, and the most common hemorrhagic site in the stomach was the antrum (*n* = 21, 43.8%). The most frequent method of hemostasis was combination therapy (*n* = 32, 44.4%). The incidence of rebleeding was 37.5% (*n* = 27), and 16.7% (*n* = 12) of patients expired due to hemorrhage. In a multivariate analysis of the risk factors for rebleeding, alcoholism (OR = 11.19, *P* = 0.02), the experience of endoscopists (OR = 0.56, *P* = 0.03), and combination endoscopic therapy (OR = 0.06, *P* = 0.01) compared with monotherapy were significantly related to rebleeding after endoscopic therapy. In a risk analysis of mortality after endoscopic therapy, only rebleeding was related to mortality (OR = 7.1, *P* = 0.02).

CONCLUSION: Intensive combined endoscopic treatments by experienced endoscopists are necessary for the treatment of NGIH in patients with CKD, especially when a patient is an alcoholic.

Key words: Chronic kidney diseases; Gastrointestinal hemorrhage; Endoscopy; Peptic ulcer; Alcoholics

Core tip: Patients with chronic kidney disease (CKD) have increased hemorrhagic complications, including nonvariceal upper gastrointestinal hemorrhage (NGIH). These individuals also have a higher risk of rebleeding than patients without renal dysfunction. Initial intensive

combined endoscopic treatments by experienced endoscopists are necessary for the treatment of NGIH in patients with CKD, especially when a patient is an alcoholic. Factors of the consumption of alcohol, endoscopic monotherapy, and endoscopists' lack of experience are associated with rebleeding, which is the most important factor for the prediction of mortality in CKD patients with NGIH.

Bang CS, Lee YS, Lee YH, Sung H, Park HJ, Kim HS, Kim JB, Baik GH, Kim YS, Yoon JH, Kim DJ, Suk KT. Characteristics of nonvariceal upper gastrointestinal hemorrhage in patients with chronic kidney disease. *World J Gastroenterol* 2013; 19(43): 7719-7725 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7719.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7719

INTRODUCTION

The hemostasis rate of nonvariceal upper gastrointestinal hemorrhage (NGIH) is 90%[1]. However, rebleeding develops in approximately 20% of patients, and the mortality rate has been reported as ranging from 6%-25%[1-8]. In patients with chronic kidney disease (CKD), gastrointestinal hemorrhage is a common complication[9], and hemorrhage from the upper gastrointestinal tract from these patients accounts for 7.8%-12.2% of patients with total upper gastrointestinal hemorrhage[10]. Peptic ulcer is the most common cause of upper gastrointestinal hemorrhage in CKD, followed by erosive gastritis, esophagitis, vascular ectasia, and angiodysplasia[9-12]. Several reports have suggested that the prevalence of peptic ulcer in patients with CKD is higher than in the general population[13,14]. Many studies on the outcome of and risk factors for peptic ulcer bleeding in patients with normal renal function have been reported. However, there are few studies on the outcome of acute hemorrhage due to peptic ulcer and the risk factors for rebleeding in patients with CKD[10,12].

Although the pathogenesis of hemorrhage in patients with CKD is not completely understood, three hypotheses have been proposed to explain the mechanism. First, uremic platelet dysfunction is believed to be the most important factor[12,15]. Second, a high rate of platelet dysfunction may be responsible for the increased frequency of rebleeding compared with the frequency in patients without renal dysfunction[9,16,17]. Lastly, previous studies on peptic ulcer and upper gastrointestinal hemorrhage in patients with renal dysfunction have suggested that hemorrhage in these patients is associated with acid secretion and mucosal integrity[11,18-20]. Moreover, hemodialysis, heparin use, abnormal platelet function, and anemia could be related to NGIH in patients with end-stage renal disease (ESRD), although the evidence is limited. Thus, the objective of this study was to evaluate the clinical characteristics of upper gastrointestinal ulcer hemorrhage in CKD patients and to determine the risk factors for rebleeding in patients undergoing endoscopic therapy.

MATERIALS AND METHODS

Patients

Between December 2003 and December 2010, a total of 72 CKD patients (M:F = 52:20, mean age: 63.9 ± 11.1) who had undergone endoscopic therapy for NGIH were retrospectively evaluated. CKD was defined according to the National Kidney Foundation Kidney Disease Outcomes Quality Initiative guidelines for nephrologists, which describe the presence of either kidney damage or decreased renal function (glomerular filtration rate < 60 mL/min per 1.73 m^2) for more than 3 mo. ESRD was defined as chronic kidney failure (glomerular filtration rate < 15 mL/min per 1.73 m^2) treated by dialysis. Patients who were not treated with endoscopic management and who had mallory-weiss syndrome were excluded. The clinical and endoscopic characteristics of patients with rebleeding were reviewed and compared with the characteristics of patients without rebleeding. Data were recorded for the following variables: gender, age, dialysis use, the method of dialysis, alcohol use and smoking history, past hemorrhage history, endoscopic findings (the cause, location, and size of the hemorrhage and the hemorrhagic state), endoscopic therapy, endoscopist experience, clinical outcomes, and mortality. Written informed consent to treatment was obtained from each patient. The study was performed in accordance with the Helsinki Declaration.

The patients' vital signs were checked every 10 min before endoscopy, every 2 h for the 24 h after endoscopy, and every 6 h after follow-up endoscopy. The hemoglobin level was checked more than once per day, and a blood transfusion was performed if the hemoglobin level decreased to below 9 g/dL. Rebleeding was defined as fresh hematemesis, fresh melena with blood pressure < 100 mmHg, a drop in the hemoglobin level of more than 2 g/dL, or endoscopic confirmation of hemorrhage or pathologic lesions necessitating endoscopic management within 7 d after initial therapy. The hemorrhagic state was classified into five groups based on Forrest's classification: active pumping, active oozing, vessel exposure, red clots, and black clots.

Endoscopic therapy

All of the patients presented to the hospital with NGIH and underwent an endoscopic examination within 24 h. Endoscopic management for peptic ulcer bleeding was performed. Intravenous proton pump inhibitors (PPIs) were prescribed to promote healing of the lesion before and after endoscopic therapy. Levin tube irrigation with more than 3000 cc of normal saline was performed before initial endoscopy. Thirteen experienced gastroenterologists (> 6000 cases of endoscopy) performed therapeutic endoscopic procedures for NGIH during the study period. The therapeutic options used to treat NGIH were

Table 1 Clinical characteristics of chronic kidney disease patients with peptic ulcer hemorrhage

Variables	Number
Gender (Male/female)	52/20
Age (yr)[1]	63.9 ± 11.1
Alcohol (Yes/no)	25/47
Smoking (Yes/no)	29/43
Past hemorrhage History (Yes/no)	11/61
ESRD (Yes/no)	50/22
Dialysis	
Hemodialysis	45
Peritoneal dialysis	5
Symptoms	32/28/2/10
(Hematemesis/Melena/Syncope/Others)	
Initial blood pressure	
Systolic (mmHg)[1]	128.7 ± 40.0
Diastolic (mmHg)[1]	75.9 ± 16.5
Initial heart rates[1]	94.0 ± 24.5
Hb (g/dL)[1]	7.4 ± 2.0
Platelet (10^9/L)[1]	208 ± 156
INR/PTT (second)[1]	1.14 ± 0.35/47.2 ± 55.6

[1]mean ± SD. ESRD: End-stage renal disease; INR: International normalized ratio; Hb: Haemoglobin.

1:10000 epinephrine injection, fibrin glue (Greenplast, Green Cross, Chung won, South Korea) injection, hemoclipping, electrocoagulation, endoscopic band ligation, and argon plasma coagulation. The patients were scheduled for a follow-up endoscopic examination within 24 h. If their general condition was not suitable for endoscopy, the follow-up examination was delayed. At the follow-up examination, oral intake was initiated, and endoscopic biopsy and *Helicobacter pylori* (*H. pylori*) tests were performed. If active bleeding or vessel exposure was observed during the follow-up endoscopic examination, this event was considered as rebleeding, and second endoscopic hemostasis was attempted. If continuous bleeding developed, not controlled by endoscopic hemostasis, operational or interventional radiologic management was performed. During fasting periods, intravenous pantoprazole sodium as a 40 mg bolus was supplied twice per day. After starting oral intake, a standard dose of oral PPIs was administered every morning for 6-8 wk. If the tests for *H. pylori* were positive, eradication medications (PPI + amoxicillin + clarithromycin) were administered for 7 d. Laboratory tests, abdominal ultrasonography, and routine abdominal X-ray were performed after the procedure to evaluate possible complications, including rebleeding or perforation.

Statistical analysis

The χ^2 test and Student's *t* test were used to evaluate baseline characteristics. Categorical variables were analyzed by the χ^2 test, and continuous variables were assessed by the Student's *t* test. Univariate analysis and multivariate logistic regression were used to detect independent risk factors related to rebleeding during follow-up periods and prognosis. A *P* value < 0.05 was considered as significant for all tests. Analyses were performed using SPSS software, version 18.0 (SPSS Inc., Chicago, IL, United States).

Table 2 Endoscopic findings, therapy and prognosis of clinical risk factors patients with peptic ulcer hemorrhage

Variables	Numbers
Size of ulcer (mm)[1]	13.7 ± 10.2
Hemorrhage state	6/38/18/5/5
(Pumping/oozing/vessel/red/black)	
Location 1 (Gastric/Duodenum)	48/24
Location 2 (Antrum/Angle/Body/Cardia)	21/11/15/1
Location 3 (Anterior/Posterior/Lesser/Greater)	11/7/22/8
Endoscopic therapy	27/8/5/32
(Injection/Coagulation/Clip/Combination)	
Amount of epinephrine (cc)[1]	15.6 ± 12.9
Experience of endoscopists (yr)[1]	3.5 ± 2.7
H. pylori infection (Yes/no)	19/33
Rebleeding (Yes/no)	27/45
Hemorrhage related death (Yes/no)	12/60

[1]mean ± SD. *H. pylori*: *Helicobacter pylori*.

RESULTS

Characteristics of patients

During the 4-year study period, 72 CKD patients with peptic ulcer hemorrhage were identified. The clinical characteristics of these patients are summarized in Table 1. The mean age of the patients with CKD was 63.9 ± 11.1. In total, 61 (84.7%) patients were experiencing their first hemorrhagic episode; 8 (11.1%) patients, their second; and 3 (4.2%) patients, their third. In this study, 50 (69.4%) ESRD patients were detected, of whom 45 (90%) patients were undergoing hemodialysis, and 5 (10%) were undergoing peritoneal dialysis. The initial systolic blood pressure of the patients was 128.7 ± 40.0 mmHg, and the diastolic blood pressure was 75.9 ± 16.5 mmHg. The hemoglobin level of the patients was 7.4 ± 2.0 g/dL, and the platelet count was 208 ± 156 (10^9/L).

All of these patients were managed by endoscopy for peptic ulcer hemorrhage. The endoscopic findings and treatments of these patients are shown in Table 2. The mean ulcer size (mm) was 13.7 ± 10.2, and the hemorrhagic location the stomach in 48 (66.7%) cases and the duodenum in 24 (33.3%) cases. The most common hemorrhagic site in the stomach was the antrum (43.8%).

The therapeutic method of endoscopy was injection for 27 (37.5%) patients, coagulation for 8 (11.1%) patients, clipping for 5 (6.9%) patients, and combination therapy for 32 (44.4%) patients. The most common combination was epinephrine and glue injection (n = 11), followed by epinephrine injection and coagulation (n = 10) and epinephrine injection and clipping (n = 10). The mean number of years of experience of the endoscopists was 3.5 ± 2.7 years. The total number of patients with rebleeding was 27 (37.5%), and hemorrhage-related death was observed in 12 patients (16.7%, Table 2).

Univariate analysis of risk factors for rebleeding

The incidence of rebleeding was 37.5% (n = 27), and

Table 3 Univariate analysis for clinical risk factors of rebleeding

Characteristics	Rebleeding (n = 27)	No Rebleeding (n = 45)	P value
Gender (Male/female)	18/9	34/11	NS
Age (yr)[1]	62.9 (11.5)	64.6 (11.0)	NS
Heart rate[1]	95 (17)	93 (29)	NS
CKD (not ESRD)/ESRD	10/17	12/33	NS
Previous hemorrhage history (Yes/no)	4/23	7/38	NS
Blood pressure			
Systolic (mmHg)[1]	128 (32)	129 (45)	NS
Diastolic (mmHg)[1]	76 (16)	76 (17)	NS
Lab			
Hb (g/dL)[1]	7.4 (1.9)	7.5 (2.1)	NS
Platelet (10^9/L)[1]	207 (174)	208 (145)	NS
INR/PTT	1.2/46.7	1.1/48.0	NS
Alcohol (Yes/no)	15/12	10/35	< 0.01
Smoking (Yes/no)	15/12	14/31	< 0.05

[1]mean ± SD. NS: Not significant; CKD: Chronic kidney disease; ESRD: End-stage renal disease; INR: International normalized ratio; Hb: Haemoglobin.

Table 4 Univariate analysis for endoscopic risk factors of rebleeding

Variables	Rebleeding	No rebleeding	P value
Hemorrahge state			NS
Active pumping	1	5	
Active oozing	15	23	
Blood vessel	7	11	
Red or black clot	4	6	
Ulcer size (mm)[1]	14.4 (11.9)	13.2 (9.1)	NS
Location (antrum and angle *vs* body)	11/7	21/9	NS
H. pylori infection (Yes/no)	7/20	17/28	NS
Therapy			NS
Injection	11	16	
APC or electrocoagulation	4	4	
Clip	2	3	
Combination	10	22	
Amount epinephrine[1]	16.9 (15.3)	14.7 (11.3)	NS
Endoscopists' experience[1]	2.8 (1.9)	4.0 (3.0)	< 0.05
Endoscopists' status			< 0.05
Fellowship doctor	20	19	
Faculty doctor	7	26	

[1]mean ± SD. NS: Not significant; *H. pylori*: *Helicobacter pylori*; APC: Argon plasma coagulation

16.7% (n = 12) of patients expired due to bleeding. In the univariate analysis of clinical risk factors for rebleeding, there was no statistically significant difference in gender, age, dialysis method, or previous hemorrhage history between the rebleeding and the no-rebleeding groups (Table 3). Alcohol consumption was noted for 15/27 (55.6%) patients in the rebleeding group and 10/45 (22.2%) patients in the no-rebleeding group (P < 0.01). Additionally, smoking was reported by 15/27 (55.6%) patients in the rebleeding group and 14/45 (31.1%) patients in the no-rebleeding group (P < 0.01). The univariate analysis of endoscopic risk factors for rebleeding is shown in Table 4. Hemorrhagic states, ulcer sizes, and therapeutic methods were not significantly different between the rebleeding and the no-rebleeding groups. However, the number of years of experience of the endoscopists was 2.8 years for the rebleeding group and 4.0 years for the no-rebleeding group, which was a statistically significant difference (P < 0.05). In an analysis according to the endoscopists' status, being a doctor on fellowship was associated with rebleeding compared with being a doctors on the faculty (OR = 2.1, P = 0.02).

Multivariate analysis of risk factors for rebleeding and mortality

In the multivariate analysis of risk factors for rebleeding, the consumption of alcohol, endoscopic monotherapy, and endoscopists' lack of experience were associated with rebleeding development (Table 5). The alcohol-consuming group had an OR of 11.19 (P = 0.02) for rebleeding compared with the non-alcohol-consuming group. Although therapeutic methods were not associated with rebleeding in the univariate analysis, combination endoscopic treatment was associated with less frequent development of rebleeding in the multivariate analysis (OR = 0.06, P = 0.01). The experience of endoscopists was significantly associated with the development of rebleeding in the multivariate analysis (OR = 0.56, P = 0.03). The risk factor associated with prognosis was rebleeding alone (OR = 7.10, P = 0.02, Table 6).

DISCUSSION

Patients with CKD have increased hemorrhagic complications[10]. Additionally, there are a higher rebleeding risk and greater mortality in patients on dialysis than in patients without renal dysfunction[11]. The current study investigated peptic ulcer hemorrhage in CKD patients and found that these individuals are at high risk of rebleeding. In this study, the rebleeding rate in patients with CKD was 37.5%. This result is higher than for the CKD (14%) or normal renal function (12%) group and similar to rebleeding in ESRD patients (38%) in a previous study[12]. Moreover, the result is higher than the rate determined for Korean CKD patients (14.3%) in another study[21]. However, the definition of rebleeding differed. In the present study, rebleeding was confined to episodes within 7 d after endoscopic therapy, whereas there was no clear statement about time in previous studies, in which even rebleeding at 30 d after the first hemorrhage was included[12]. Taking these results together, CKD patients have a higher risk of rebleeding than patients without renal dysfunction, with approximately more than one third of CKD patients experiencing rebleeding.

NGIH is associated with high mortality. The mortality of NGIH complicating acute renal dysfunction is 68.3% and of NGIH complicating severe liver cirrhosis (LC) is 68.4%[22,23]. In the present study, the mortality of CKD patients with NGIH was 16.7%. This result is higher than the 13% value determined in a Taiwanese study and

Table 5 Multivariate analysis for risk factors of rebleeding

Variables	*P* value	OR
Age	NS	
Gender	NS	
Smoking	NS	
Hb (g/dL)	NS	
Platelet (10^9/L)	NS	
INR	NS	
Ulcer size	NS	
Location	NS	
Hemorrhage state	NS	
Amount of epinephrine	NS	
Alcohol (Yes/no[1])	0.02	11.19
Therapy (Combination therapy/monotherap[1])	0.01	0.06
Experiences of endoscopists (yr)	0.03	0.56

[1]Reference category. NS: Not significant; INR: International ormalized ratio; Hb: Haemoglobin B.

Table 6 Multivariate analysis for risk factors of prognosis

Variables	*P* value	OR
Age	NS	1.07
Gender (Male/female[1])	NS	1.87
Hemorrhage state	NS	
Active pumping		0.07
Active oozing		0.00
Blood vessel		0.17
Red clot		0.00
Black clot[1]		0.77
Rebleeding (Yes/no[1])	0.02	7.10

[1]Reference category. NS: Not significant.

the 8.6% value reported in another Korean study[10,21]. In particular, rebleeding was related to the mortality of the CKD patients (OR = 7.1, $P = 0.02$). According to a study that used nationwide inpatient samples from the United States, mechanical ventilation, severe sepsis, disseminated intravascular coagulation, cancer, age (> 65 years), coagulation defects, and venous thromboembolism were predictors of mortality in patients with ESRD and NGIH[24]. However, that study did not include rebleeding in the statistical analysis, although other studies have emphasized the importance of this parameter[12,21,24]. Overall, rebleeding is the most important factor for the prediction of mortality in CKD patients with NGIH, and the prevention of rebleeding should be a goal of clinical practice.

Regarding risk factors for rebleeding, the experience of endoscopists was one of the main factors (OR = 0.56, $P = 0.03$). In a retrospective study in Canada, ESRD itself and ulcer with high-risk stigmata were the factors associated with rebleeding[12]. However, the experience of endoscopists was not analyzed. According a study of risk factors for rebleeding in NGIH patients, regardless of renal function, lower hemoglobin levels, endoscopist inexperience (< 2 years of experience), and comorbidity with CKD or LC were the associated factors[25]. These results indicate that insufficient or inappropriate endoscopic management by inexperienced endoscopists could result in rebleeding in CKD patients with NGIH. Moreover, considering that emergency endoscopic hemostasis procedures are frequently performed at night or on holidays by only one endoscopist, without the help of colleagues, intensive endoscopic treatments are important.

Combined endoscopic management was associated with a reduced risk of rebleeding in this study (*vs* monotherapy, OR = 0.06, $P = 0.01$). Recent studies have suggested that combined endoscopic hemostasis treatments are superior to single treatments[26,27]. In a study of the risk factors for rebleeding in NGIH patients, regardless of renal function, combination therapy (injection + thermal therapy) was associated with lower mortality compared with injection therapy alone[25]. These results are consistent with the findings of our study. Among the endoscopic therapeutic options, injection therapy ($n = 27$, 67.5%) was preferred over thermal ablation ($n = 8$, 20%), such as argon plasma coagulation or electrocoagulation, when used as single method in this study. This preference was due to concerns about tissue injury or loss in thermal therapy, which could result in rebleeding in patients with a hemorrhagic tendency. Another finding of this study was that being an endoscopic doctor on fellowship was associated with rebleeding compared with being a doctor on the faculty (OR = 2.1, $P = 0.02$). Overall, intensive endoscopic treatment by experts using a combined method can result in better outcomes in CKD patients with NGIH.

Another finding of this study was related to alcohol use. In the multivariate analysis of risk factors for rebleeding, alcohol use was demonstrated to be an important risk factor (OR = 11.19, $P = 0.02$). Additionally, the most common hemorrhagic site was the lesser curvature of the antrum of the stomach. *H. pylori* infection was detected in only 33.3% of patients. The exact mechanism underlying the association of alcohol use and *H. pylori* with NGIH in patients with CKD is not understood. However, endoscopists have to pay more attention when a patient is an alcoholic because of rebleeding risk, which is associated with high mortality.

There are several limitations of this study. First, it was retrospective study, and a small number of patients were evaluated. Second, hospital stay and blood transfusion, which were evaluated in other studies[12,28], were not assessed. Further studies are needed to improve outcomes in CKD patients with NGIH.

In conclusion, initial intensive combined endoscopic treatments by experienced endoscopists are necessary for the treatment of NGIH in patients with CKD, especially when a patient is an alcoholic, as rebleeding after endoscopic treatment is a risk factor for mortality.

COMMENTS

Background

Peptic ulcer is the most common cause of upper gastrointestinal hemorrhage in chronic kidney disease (CKD). Additionally, there are a higher rebleeding risk and greater mortality in patients on dialysis than in patients without renal dysfunction. Many studies on the outcome of and risk factors for peptic ulcer

bleeding in patients with normal renal function have been reported. However, there have been few studies on the outcome of acute hemorrhage due to peptic ulcer and on the risk factors for rebleeding in patients with CKD.

Research frontiers

According to a study that used nationwide inpatient samples from the United States, mechanical ventilation, severe sepsis, disseminated intravascular coagulation, cancer, age (> 65 years), coagulation defects, and venous thromboembolism were predictors of mortality in patients with end-stage renal disease and nonvariceal upper gastrointestinal hemorrhage (NGIH).

Innovations and breakthroughs

In the previous study on predictors of mortality in patients with ESRD and NGIH, rebleeding was not determined to be a major predictor of mortality. However, only rebleeding was related to mortality (OR = 7.1, P = 0.02) in the current study. Moreover, alcoholism (OR = 11.19, P = 0.02), the experience of endoscopists (OR = 0.56, P = 0.03), and combination endoscopic therapy (OR = 0.06, P = 0.01) compared with monotherapy were significantly related to rebleeding after endoscopic therapy in this study.

Applications

Initial intensive combined endoscopic treatments by experienced endoscopists are necessary for the treatment of NGIH in patients with CKD, especially when a patient is an alcoholic. These factors are associated with rebleeding, which is the most important factor for the prediction of mortality in CKD patients with NGIH.

Peer review

This is a well done study. It is an important topic. The manuscript is interesting, well done and well written.

REFERENCES

1 **Lee JH**, Kim BK, Seol DC, Byun SJ, Park KH, Sung IK, Park HS, Shim CS. Rescue endoscopic bleeding control for nonvariceal upper gastrointestinal hemorrhage using clipping and detachable snaring. *Endoscopy* 2013; **45**: 489-492 [PMID: 23580408 DOI: 10.1055/s-0032-1326375]

2 **Laine L**, Peterson WL. Bleeding peptic ulcer. *N Engl J Med* 1994; **331**: 717-727 [PMID: 8058080 DOI: 10.1056/nejm199409153311107]

3 **Müller T**, Barkun AN, Martel M. Non-variceal upper GI bleeding in patients already hospitalized for another condition. *Am J Gastroenterol* 2009; **104**: 330-339 [PMID: 19174801 DOI: 10.1038/ajg.2008.62]

4 **Chiu PW**, Joeng HK, Choi CL, Kwong KH, Ng EK, Lam SH. Predictors of peptic ulcer rebleeding after scheduled second endoscopy: clinical or endoscopic factors? *Endoscopy* 2006; **38**: 726-729 [PMID: 16810597 DOI: 10.1055/s-2006-925179]

5 **Lin HJ**, Lo WC, Lee FY, Perng CL, Tseng GY. A prospective randomized comparative trial showing that omeprazole prevents rebleeding in patients with bleeding peptic ulcer after successful endoscopic therapy. *Arch Intern Med* 1998; **158**: 54-58 [PMID: 9437379]

6 **Wolf AT**, Wasan SK, Saltzman JR. Impact of anticoagulation on rebleeding following endoscopic therapy for nonvariceal upper gastrointestinal hemorrhage. *Am J Gastroenterol* 2007; **102**: 290-296 [PMID: 17100959 DOI: 10.1111/j.1572-0241.2006.00969.x]

7 **Tajima A**, Koizumi K, Suzuki K, Higashi N, Takahashi M, Shimada T, Terano A, Hiraishi H, Kuwayama H. Proton pump inhibitors and recurrent bleeding in peptic ulcer disease. *J Gastroenterol Hepatol* 2008; **23** Suppl 2: S237-S241 [PMID: 19120905 DOI: 10.1111/j.1440-1746.2008.05557.x]

8 **Andriulli A**, Annese V, Caruso N, Pilotto A, Accadia L, Niro AG, Quitadamo M, Merla A, Fiorella S, Leandro G. Proton-pump inhibitors and outcome of endoscopic hemostasis in bleeding peptic ulcers: a series of meta-analyses. *Am J Gastroenterol* 2005; **100**: 207-219 [PMID: 15654802 DOI: 10.1111/j.1572-0241.2005.40636.x]

9 **Zuckerman GR**, Cornette GL, Clouse RE, Harter HR. Upper gastrointestinal bleeding in patients with chronic renal failure. *Ann Intern Med* 1985; **102**: 588-592 [PMID: 3872616]

10 **Tsai CJ**, Hwang JC. Investigation of upper gastrointestinal hemorrhage in chronic renal failure. *J Clin Gastroenterol* 1996; **22**: 2-5 [PMID: 8776085]

11 **Boyle JM**, Johnston B. Acute upper gastrointestinal hemorrhage in patients with chronic renal disease. *Am J Med* 1983; **75**: 409-412 [PMID: 6604455]

12 **Cheung J**, Yu A, LaBossiere J, Zhu Q, Fedorak RN. Peptic ulcer bleeding outcomes adversely affected by end-stage renal disease. *Gastrointest Endosc* 2010; **71**: 44-49 [PMID: 19595311 DOI: 10.1016/j.gie.2009.04.014]

13 **Luo JC**, Leu HB, Huang KW, Huang CC, Hou MC, Lin HC, Lee FY, Lee SD. Incidence of bleeding from gastroduodenal ulcers in patients with end-stage renal disease receiving hemodialysis. *CMAJ* 2011; **183**: E1345-E1351 [PMID: 22083684 DOI: 10.1503/cmaj.110299]

14 **Wu CY**, Wu MS, Kuo KN, Wang CB, Chen YJ, Lin JT. Long-term peptic ulcer rebleeding risk estimation in patients undergoing haemodialysis: a 10-year nationwide cohort study. *Gut* 2011; **60**: 1038-1042 [PMID: 21266725 DOI: 10.1136/gut.2010.224329]

15 **Weigert AL**, Schafer AI. Uremic bleeding: pathogenesis and therapy. *Am J Med Sci* 1998; **316**: 94-104 [PMID: 9704663]

16 **Remuzzi G**, Livio M, Marchiaro G, Mecca G, de Gaetano G. Bleeding in renal failure: altered platelet function in chronic uraemia only partially corrected by haemodialysis. *Nephron* 1978; **22**: 347-353 [PMID: 740096]

17 **Remuzzi G**, Benigni A, Dodesini P, Schieppati A, Gotti E, Livio M, Mecca G, Donati B, de Gaetano G. Platelet function in patients on maintenance hemodialysis: depressed or enhanced? *Clin Nephrol* 1982; **17**: 60-63 [PMID: 7067167]

18 **Sullivan SN**, Tustanoff E, Slaughter DN, Linton AL, Lindsay RM, Watson WC. Hypergastrinemia and gastric acid hypersecretion in uremia. *Clin Nephrol* 1976; **5**: 25-28 [PMID: 765028]

19 **Goldstein H**, Murphy D, Sokol A, Rubini ME. Gastric acid secretion in patients undergoing chronic dialysis. *Arch Intern Med* 1967; **120**: 645-653 [PMID: 6058399]

20 **Ventkateswaran PS**, Jeffers A, Hocken AG. Gastric acid secretion in chronic renal failure. *Br Med J* 1972; **4**: 22-23 [PMID: 5078407]

21 **Kim HK**, Kim YS, Cho YS, Ji JS, Kim SS, Kim YO, Yun S, Chae HS, Choi KY, Chung IS. Clinical Features of Gastrointestinal Bleeding in Patients with Chronic Renal Failure. *Korean J Gastrointestinal Endoscopy* 2006; **32**: 374-380

22 **Fiaccadori E**, Maggiore U, Clima B, Melfa L, Rotelli C, Borghetti A. Incidence, risk factors, and prognosis of gastrointestinal hemorrhage complicating acute renal failure. *Kidney Int* 2001; **59**: 1510-1519 [PMID: 11260415 DOI: 10.1046/j.1523-1755.2001.0590041510.x]

23 **Chen YC**, Tsai MH, Hsu CW, Ho YP, Lien JM, Chang MY, Fang JT, Huang CC, Chen PC. Role of serum creatinine and prognostic scoring systems in assessing hospital mortality in critically ill cirrhotic patients with upper gastrointestinal bleeding. *J Nephrol* 2003; **16**: 558-565 [PMID: 14696759]

24 **Sood P**, Kumar G, Nanchal R, Sakhuja A, Ahmad S, Ali M, Kumar N, Ross EA. Chronic kidney disease and end-stage renal disease predict higher risk of mortality in patients with primary upper gastrointestinal bleeding. *Am J Nephrol* 2012; **35**: 216-224 [PMID: 22310659 DOI: 10.1159/000336107]

25 **Suk KT**, Kim HS, Lee CS, Lee IY, Kim MY, Kim JW, Baik SK, Kwon SO, Lee DK, Ham YL. Clinical outcomes and risk factors of rebleeding following endoscopic therapy for nonvariceal upper gastrointestinal hemorrhage. *Clin Endosc* 2011; **44**: 93-100 [PMID: 22741119 DOI: 10.5946/ce.2011.44.2.93]

26 **Marmo R**, Rotondano G, Bianco MA, Piscopo R, Prisco A, Cipolletta L. Outcome of endoscopic treatment for peptic ulcer bleeding: Is a second look necessary? A meta-analysis. *Gastrointest Endosc* 2003; **57**: 62-67 [PMID: 12518133 DOI:

10.1067/mge.2003.48]
27 **Calvet X**, Vergara M, Brullet E, Gisbert JP, Campo R. Addition of a second endoscopic treatment following epinephrine injection improves outcome in high-risk bleeding ulcers. *Gastroenterology* 2004; **126**: 441-450 [PMID: 14762781]
28 **Toke AB**. GI bleeding risk in patients undergoing dialysis. *Gastrointest Endosc* 2010; **71**: 50-52 [PMID: 20105475 DOI: 10.1016/j.gie.2009.09.005]

P- Reviewers: Grizzi F, Quigley EMM **S- Editor:** Cui XM
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.v19.i43.7726

World J Gastroenterol 2013 November 21; 19(43): 7726-7734
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Events associated with apoptotic effect of *p*-Coumaric acid in HCT-15 colon cancer cells

Saravana Kumar Jaganathan, Eko Supriyanto, Mahitosh Mandal

Saravana Kumar Jaganathan, Eko Supriyanto, IJ N-UTM Cardiovascular Engineering Centre, Faculty of Bioscience and Medical Engineering, Universiti Teknologi Malaysia, Johor Bahru 81310, Malaysia
Saravana Kumar Jaganathan, Department of Research and Development, PSNA College of Engineering and Technology, Dindigul 624622, India
Mahitosh Mandal, School of Medical Science and Technology, Indian Institute of Technology, Kharagpur 721302, West Bengal, India
Author contributions: Jaganathan SK designed and performed the research experiments; Jaganathan SK, Supriyanto E analyzed the data; Jaganathan SK wrote the paper; Mandal M contributed the new reagents for the study; Supriyanto E and Mandal M provided suggestions and edited the paper.
Supported by Universiti Teknologi Malaysia, Malaysia for providing Visiting Research Fellowship
Correspondence to: Dr. Saravana Kumar Jaganathan, IJ N-UTM Cardiovascular Engineering Centre, Faculty of Bioscience and Medical Engineering, Universiti Teknologi Malaysia, Johor Bahru 81310, Malaysia. jaganathaniitkgp@gmail.com
Telephone: +91-607-5558548 Fax: +91-607-5558553
Received: May 14, 2013 Revised: July 24, 2013
Accepted: August 20, 2013
Published online: November 21, 2013

Abstract

AIM: To investigate the events associated with the apoptotic effect of *p*-Coumaric acid, one of the phenolic components of honey, in human colorectal carcinoma (HCT-15) cells.

METHODS: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertazolium-bromide assay was performed to determine the antiproliferative effect of *p*-Coumaric acid against colon cancer cells. Colony forming assay was conducted to quantify the colony inhibition in HCT 15 and HT 29 colon cancer cells after *p*-Coumaric acid treatment. Propidium Iodide staining of the HCT 15 cells using flow cytometry was done to study the changes in the cell cycle of treated cells. Identification of apoptosis was done using scanning electron microscope and photomicrograph evaluation of HCT 15 cells after exposing to *p*-Coumaric acid. Levels of reactive oxygen species (ROS) of HCT 15 cells exposed to *p*-Coumaric acid was evaluated using 2′, 7′-dichlorfluorescein-diacetate. Mitochondrial membrane potential of HCT-15 was assessed using rhodamine-123 with the help of flow cytometry. Lipid layer breaks associated with *p*-Coumaric acid treatment was quantified using the dye merocyanine 540. Apoptosis was confirmed and quantified using flow cytometric analysis of HCT 15 cells subjected to *p*-Coumaric acid treatment after staining with YO-PRO-1.

RESULTS: Antiproliferative test showed *p*-Coumaric acid has an inhibitory effect on HCT 15 and HT 29 cells with an IC_{50} (concentration for 50% inhibition) value of 1400 and 1600 μmol/L respectively. Colony forming assay revealed the time-dependent inhibition of HCT 15 and HT 29 cells subjected to *p*-Coumaric acid treatment. Propidium iodide staining of treated HCT 15 cells showed increasing accumulation of apoptotic cells (37.45 ± 1.98 *vs* 1.07 ± 1.01) at sub-G_1 phase of the cell cycle after *p*-Coumaric acid treatment. HCT-15 cells observed with photomicrograph and scanning electron microscope showed the signs of apoptosis like blebbing and shrinkage after *p*-Coumaric acid exposure. Evaluation of the lipid layer showed increasing lipid layer breaks was associated with the growth inhibition of *p*-Coumaric acid. A fall in mitochondrial membrane potential and increasing ROS generation was observed in the *p*-Coumaric acid treated cells. Further apoptosis evaluated by YO-PRO-1 staining also showed the time-dependent increase of apoptotic cells after treatment.

CONCLUSION: These results depicted that *p*-Coumaric acid inhibited the growth of colon cancer cells by inducing apoptosis through ROS-mitochondrial pathway.

Key words: Honey; Apoptosis; Rhodamine-123; Sub-G1; Merocyanine; *p*-Coumaric acid; Reactive oxygen species

Core tip: This article describes apoptotic effect of *p*-Coumaric acid, one of the phenolic components of honey, against colon cancer cells. *p*-Coumaric acid treatment resulted in the inhibition of proliferation and colony forming ability of human colorectal carcinoma (HCT-15) and HT 29 cells. Major events associated with growth-inhibition are increasing reactive oxygen species generation, increasing lipid layer breaks and a fall in Mitochondrial membrane potential. Further, membrane blebbing and shrinkage of *p*-Coumaric acid exposed HCT 15 cells insinuated apoptosis. Hence our results depicted that *p*-Coumaric acid is a prospective candidate for chemoprevention of colon cancer.

Jaganathan SK, Supriyanto E, Mandal M. Events associated with apoptotic effect of p-Coumaric acid in HCT-15 colon cancer cells. *World J Gastroenterol* 2013; 19(43): 7726-7734 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7726.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7726

INTRODUCTION

Phenolic compounds are present in various dietary agents. Consumption of such agents has been linked to improve various disease conditions like cancer, diabetes and cardiac disorders. Diet is believed to be much influential in explaining the susceptibility to cancer. Most interestingly, colon cancer is more vulnerable to diet because these epithelial cells are chronically exposed to these dietary agents[1,2]. Since, cancer of colon is among the most common malignancy among the Western and Asian nations, research communities explore various new dietary agents rich in phenolic compounds to purge this malignancy.

In our laboratory, experiments in studying the preventive effect of honey against colon cancer had been constantly done. Previous results depicted honey could inhibit the colon cancer cell proliferation. Antiproliferative effect was found to vary with the phenolic content present in the honey[3-5]. Since honey containing higher phenolic content was found to induce apoptosis significantly, the scope of this research was extended to study the apoptosis induced by one of the phenolic components of honey, *p*-Coumaric acid, against the colon cancer cells.

p-Coumaric acid is the abundant isomer of cinnamic acid and also widely found in edible plants such as peanuts, tomatoes, carrots etc. *p*-Coumaric acid is reported to have antitumor and anti-mutagenic activities[6,7]. In a study, *p*-Coumaric acid along with the combination of hydrocaffeic acid found to reduce the UV-B oxidation damage in human conjunctival cells *in vitro* and in cornea and sclera of rabbits *in vivo*[8]. In one of the latest studies, the ability of *p*-Coumaric acid to protect rat's heart against doxorubicin (DOX)-induced oxidative stress was investigated. It showed that *p*-Coumaric acid could reduce the DOX-induced high serum levels of lactic dehydrogenase and creatine phosphokinase[9]. In one of the most recent studies, effect of *p*-Coumaric acid against the colonic epithelial cells (Caco-2) was studied. *p*-Coumaric acid at a concentration of 1500 μmol/L was found to inhibit the proliferation of Caco-2 cells by 43%-75% after 24-72 h of treatment[10]. However, literature available does not depict the mechanism of *p*-Coumaric acid induced apoptosis in colon cancer cells.

Apoptosis is the major form of cell death accompanied by morphological changes like membrane blebbing and shrinkage of cells. Further, events like nuclear and chromatin condensation, DNA fragmentation and segregation of apoptotic bodies were the characteristic features of apoptosis. Reactive oxygen species (ROS) is involved in various biochemical functions like cell proliferation and apoptosis. Recent studies reported ROS mediated apoptosis is accompanied with the loss of mitochondrial membrane potential[11,12].

This current study, deals with the growth inhibitory effect of *p*-Coumaric acid in colon cancer cells. Further, an attempt has been made to explore the ROS and mitochondrial dependent mechanism in the apoptosis induced by the *p*-Coumaric acid.

MATERIALS AND METHODS

Reagents

DMEM, RPMI-1640, fetal bovine serum (FBS), *L*-glutamine, sodium pyruvate, nonessential amino acids, vitamin solution, penicillin and streptomycin were obtained from Life Technologies, Inc., Grand Island, NY, United States. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tertazolium-bromide (MTT), propidium iodide, mercury orange, rhodamine-123, RNase and *p*-Coumaric acid were purchased from Sigma-Aldrich, United States. Merocyanine 540 and YO-PRO-1 were obtained from Invitrogen Inc, United States.

Cell culture

Colon carcinoma cell line HT 29 and human colorectal carcinoma (HCT-15) (Organ: Colon, Disease: Colorectal adenocarcinoma; Organism: Human; procured from National Centre for Cell science, Pune, India) was grown in DMEM and RPMI medium respectively, supplemented with 10% FBS, *L*-glutamine, penicillin, sodium pyruvate, nonessential amino acids and vitamin solution Adherent monolayer cultures of HCT 15 were maintained in T-25 flasks and incubated at 37 ℃ in 5% carbon dioxide (CO_2). The cultures were free of mycoplasma and maintained no longer than 12 wk after recovery from frozen stocks.

Cell proliferation assay

Thiazolyl blue tetrazolium bromide (MTT) assay was carried out as follows: Cells were trypsinized, counted and 1000 cells were seeded per well in 96-well plate. The following day, 100 μL of medium containing the desired

concentration of *p*-Coumaric acid was added to the appropriate wells. The cells were then kept at 37 ℃ in 5% CO_2 for the desired length of time. Control used in these experiments was untreated cells kept for 48 h. For all the experiments performed below, control cells remained untreated and kept for the same duration as the longest time-point of the respective experiment. At this point, 100 μL of (5 mg/mL) MTT reagent was added to each well, and the plate was placed at 37 ℃ in the incubator for 2 h. 200 μL of dimethyl sulfoxide was added to each well, after aspirating the supernatant. Colored formazan product was assayed spectrophotometrically at 570 nm using enzyme-linked immunosorbent assay plate reader[12].

Colony forming assay

HCT 15 and HT 29 cells were treated with *p*-Coumaric acid at a concentration of 1400 and 1600 μmol/L respectively for definite time periods (12, 24 and 48 h) and collected by trypsinization. The cells were counted and seeded again in triplicate on a 6-well tissue culture plate with 3000 cells/well. The cells were cultured for 15 d with growth media replaced after every two days. The cells were stained with 0.5% crystal violet (in methanol) and colonies were counted[12].

Cell cycle analysis

After the appropriate treatment with *p*-Coumaric acid, HCT 15 cells were washed with phosphate-buffered saline, then resuspended in 50 μg/mL propidium iodide containing 0.1% sodium citrate with 0.1% Triton X-100 for 20 min at 4 ℃. Cells were then analyzed by flow cytometry (FACScan; Becton Dickinson Immunocytometry Systems), and the sub-G_1 fraction was used as a measure of the apoptotic cells. Control used in the experiments was untreated cells kept for 48 h. Analysis was performed in linear amplification mode in case of cell cycle analysis. Remaining experiments of flow cytometry were performed in logarithmic amplification mode unless otherwise stated[13].

Estimation of ROS generation

Dichlorofluorescein-diacetate (DCFH-DA) was cleaved by the intracellular nonspecific esterase to form DCFH. DCFH are oxidized by ROS to form the fluorescent compound DCF. *p*-Coumaric acid treated cells (1400 μmol/L) were harvested using trypsin/EDTA and resuspended in PBS. Working solution (20 μmol/L) of DCFH-DA was directly added cells and then it was incubated at 37 ℃ for 15 min. Cells were washed and resuspended in PBS and kept on ice immediately before analyzing by flow cytometry[12]. This fluorescent intensity of DCF was measured and correlated with the ROS generated in the cells.

Determination of mitochondrial membrane potential

HCT 15 colon cancer cells were treated with *p*-Coumaric acid (1400 μmol/L) for different time points. Afterwards, cells were harvested and resuspended in 1 mL of rhodamine-123 (5 μg/mL) for 1 h at 37 ℃. The intensity of fluorescence from rhodamine-123 was measured by flow cytometry[12].

Detection of membrane lipid organization

Colon cancer cells (HCT 15) were treated with *p*-Coumaric acid (1400 μmol/L) for different time points. Cells were harvested and re-suspended in 1 mL of merocyanine 540 (10 μg/mL) for 15 min at 37 ℃. The intensity of fluorescence was measured by flow cytometry[13].

YO-PRO-1 staining

YO-PRO-1 permits analysis of apoptotic cells without interfering cell viability. After treatment with *p*-Coumaric acid (1400 μmol/L), the cell pellets were mixed in 1 μmol/L YO-PRO-1 for 20 min at room temperature. After incubation intensity was measured using flow cytometry[13].

Scanning electron microscope and photomicrograph images

Fixed amount of HCT 15 cells were seeded in a sterilized glass slide and incubated for 24 h. *p*-Coumaric acid at a concentration of 1400 μmol/L was added for 48 h time interval. After incubation, cells were harvested by using trypsin/EDTA and centrifuged for 5 min at room temperature. Then the supernatant was decanted and pellet was dried. Pellet was treated with 2.5% glutaraldehyde in distilled water for 45 min in hybrid oven shaker at 37 ℃. Cells were washed thrice with PBS for 5 min and then dehydrated by ethyl alcohol of different concentration (30%, 50%, 70%, 95% and 100%) for 5-10 min. Fixing of cells was done with hexamethyl disilazane and the sample was taken for scanning electron microscope analysis. Photomicrograph images of HCT 15 and HT 29 cells were acquired using microscope.

Statistical analyses

All values are expressed as the mean ± SE. Figures were plotted using Graphpad Prism software. All experiments were performed three times independently (biological triplicates). One-way ANOVA was performed to find statistical significance.

RESULTS

MTT assay

MTT assay of treated cells was performed after 48 h of treatment. Colon cancer cells (HCT 15 and HT 29) growth was inhibited in a dose-dependent manner. Both HCT-15 and HT-29 cell growth were inhibited significantly with an IC_{50} of around 1400 μmol/L and 1600 μmol/L respectively (Figure 1A). HCT 15 cells were found more sensitive to *p*-Coumaric acid, however at higher concentrations both cell lines were found to be equally affected. Statistical analysis showed that *p*-Coumaric acid treatment results in significant inhibition (*P*

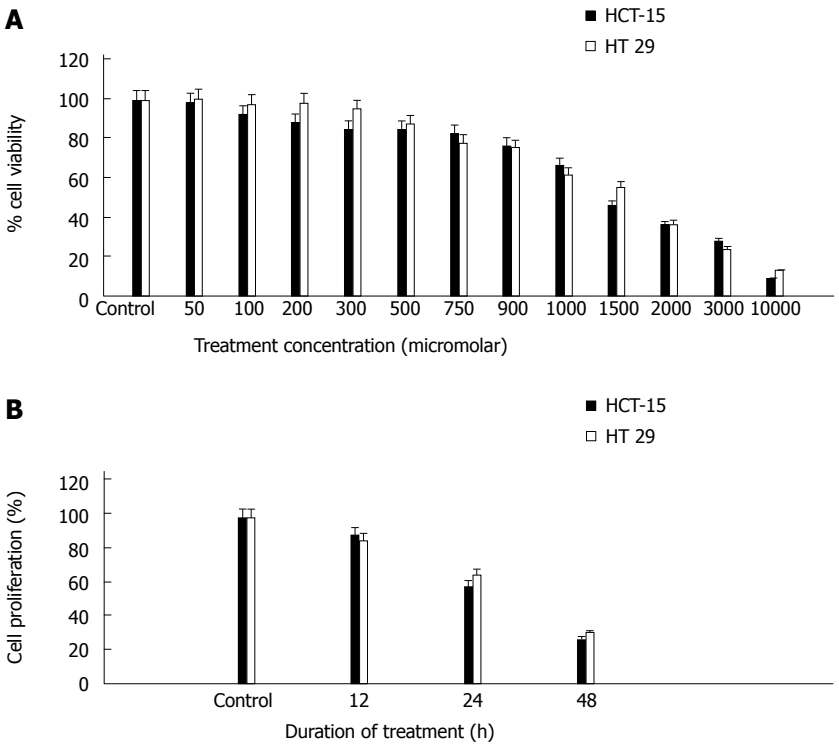


Figure 1 Antiproliferative effect, colony inhibitory of *p*-Coumaric acid against colon cancer cells. A: Both human colorectal carcinoma (HCT-15) and HT-29 cells grown in 96-well plate were treated with various concentration of *p*-Coumaric acid (0-10000 μmol/L) diluted in the media for 48 h. The mean of the percentage cell viability (% of control) along with their standard error is indicated; B: After various incubation periods of *p*-Coumaric acid treatment, colonies formed were stained with 0.5% crystal violet and counted, and percentage of survival was calculated by normalizing the values. Data reported as the mean ± SE from three different observations. Mean differences are significant at 12, 24 and 48 h.

< 0.05) compared with untreated control cells at 200 μmol/L and 500 μmol/L for HCT 15 and HT 29 cells respectively (Figure 1A).

Colony forming assay

p-Coumaric acid treated HCT 15 cells showed a maximum of 94, 67, 32 colonies after 12, 24 and 48 h of treatment. Untreated HCT 15 cells produced a maximum of 105 colonies. Similar experiment with HT29 cells displayed a maximum of 131, 101, 51 colonies after 12, 24 and 48 h treatment whereas the control HT 29 cells produced 154. A time-dependent inhibition of colony formation was clearly evident from this experiment (Figure 1B). There was a significant reduction ($P <$ 0.05) in the number of colonies formed under the various time intervals examined (both HCT 15 and HT 29 cells) when compared with corresponding untreated cells (Figure 1B).

Cell cycle analysis

Cell populations were tabulated among the sub-G_1, G_0/G_1, S and G_2/M phases of the cell cycle. It showed an increasing sub-G_1 arrest from 1.00% (control) to 37.45% after 48 h (Table 1). Statistical analysis of the sub-G1 column indicated significant increase ($P < 0.05$) of cells in the sub-G1 phase insinuating apoptosis increases with the time-dependency.

ROS generation

ROS levels were increased significantly after treatment. The increasing mean fluorescent intensity was found to be 116, 141, and 185 during 12, 24 and 48 h respectively. Untreated control cells showed an intensity of 96 after 48 h. ROS intensity after 48 h treatment was almost double the intensity of the control cells. Moreover, the differences in the ROS levels at various h examined were significant, compared to control with a P value of less than 0.05 (Figure 2).

Mitochondrial membrane potential

The decreasing mean fluorescent intensity was found to be 147, 91 during 6 and 12 h of treatment respectively. Untreated control cells showed an average intensity of 229 after 12 h. From the results, it was observed that *p*-Coumaric acid treatment reduced the potential by 2.5 fold after 12 h. There was also statistically significant reduction ($P < 0.05$) of potential at the estimated intervals compared to untreated cells (Figure 3A).

Lipid layer breaks

Untreated cells displayed a mean intensity of 33 after 6 h. Treated cells showed 37 and 50 after 3 and 6 h respectively (Figure 3B). It is evident from the above results that treated cells displayed an increase in the lipid layer breaks.

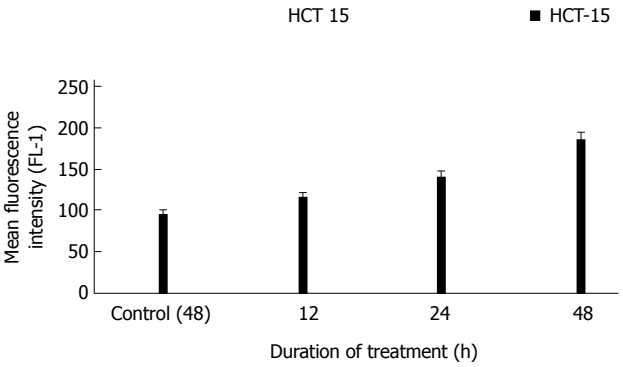


Figure 2 *p*-Coumaric acid induced reactive oxygen species generation. Human colorectal carcinoma (HCT-15) cells were cultured in the presence or absence of *p*-Coumaric acid for the specified time points. Dichlorofluorescein-diacetate fluorescence intensity was detected by using flow cytometry. Data is representative of three independent experiments and the mean differences are significant at 12, 24 and 48 h.

Table 1 Cell cycle distribution of human colorectal carcinoma-15 cells after *p*-Coumaric acid treatment

Time in h	Sub G_1[1]	G_0/G_1	S	G_2/M
Control	1.07 ± 1.01	42.82 ± 1.92	8.03 ± 1.23	40.07 ± 2.85
12 h	5.98 ± 1.17	23.06 ± 3.15	10.29 ± 4.01	46.67 ± 1.89
24 h	16.46 ± 2.03	23.92 ± 1.74	9.91 ± 3.29	39.03 ± 1.58
48 h	37.45 ± 1.98	12.79 ± 4.45	4.9 ± 3.82	17.12 ± 4.65

[1]Mean differences are significant at $P < 0.05$. Data represents mean ± SD.

Photomicrograph and scanning electron microscope images

Scanning electron microscope (SEM) images of *p*-Coumaric acid treated cells (48 h) showed typical signs of apoptosis like membrane blebbing and shrinkage as indicated by arrow marks. Normal cells were found almost spherical without marked shrinkage (Figure 4A). This was further corroborated with the photomicrograph images (Figure 4B).

Yo-Pro-1 staining

The percentage of cells distributed in M2 population signifying apoptosis increased depending upon the duration of treatment. It was found to be 20, 33 after 24 and 48 h of *p*-Coumaric acid treatment. M2 phase population of untreated control cells was found to be 8% after 48 h (Figure 5).

DISCUSSION

Diet consumption and cancer have been linked by various studies[14,15]. They postulated that consistent pattern of consumption of diets which are rich in vegetables and fruits may reduce the risk of cancer. Phenolic compounds, one of the classes of non-nutritive phytochemicals, are widely distributed in our foods and suggested to have preventive effect against various disease conditions like cancer, diabetes and several cardiac disorders[16,17].

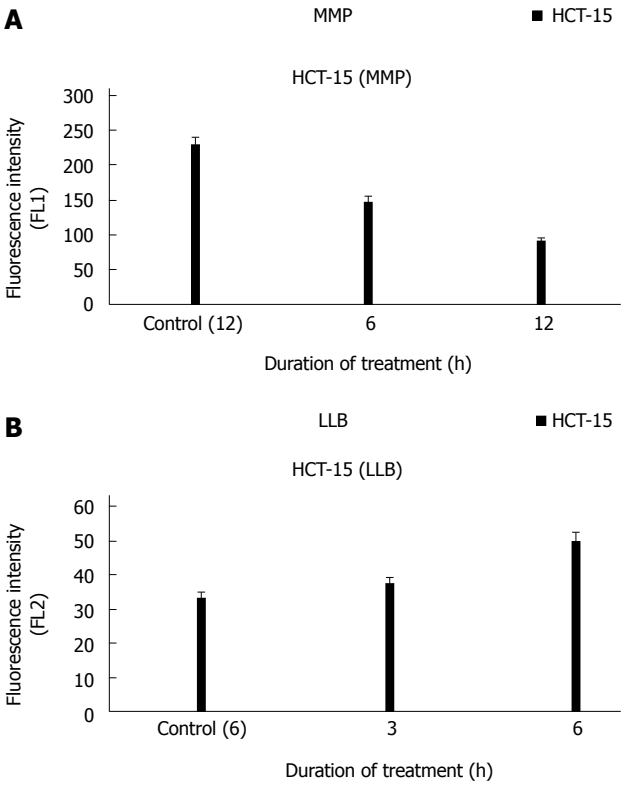


Figure 3 Events associated with growth-inhibitory effect of *p*-Coumaric acid. A: Human colorectal carcinoma (HCT-15) cells were treated with *p*-Coumaric acid for specified time-periods and then mitochondrial membrane potential were determined using rhodamine-123 by flow cytometry. Mean differences are significant at 6 and 12 h compared with untreated control cells ($P < 0.05$ *vs* untreated control cells); B: HCT 15 cells were treated with *p*-Coumaric acid and evaluated using merocyanine 540 to quantify the lipid layer breaks (LLBs). Data is representative of three independent experiments and mean differences are significant at 3 and 6 h compared with untreated control cells ($P < 0.05$ *vs* untreated control cells). MMP: Mitochondrial membrane potential.

From our laboratory, it was showed that honey rich in phenolic content was able to induce apoptosis significantly in colon cancer cells. Hence, in this research the effect of *p*-Coumaric acid, one of the phenolic constituents of honey, induced apoptosis in colon cancer cells was studied.

p-Coumaric acid inhibited the proliferation of colon cancer cells. Both HCT-15 and HT-29 cell growth were inhibited significantly with an IC_{50} of around 1400 μmol/L and 1600 μmol/L respectively. This was similar to the previously published report on the antiproliferative effect of *p*-Coumaric acid against Caco-2 cells[10]. Bioavailability of phenolic constituents is a major factor when we would like to examine the effect of *p*-coumaric acid in *in vivo*. In one of the researches, it was showed that bioavailability of coumaric acid after consumption of 200 g plum is in the range of 28-230 mg/serving[18]. In a colonic volume of 200 mL, this would yield a concentration in the range of 850 to 7000 μmol/L. Hence, it is believed that estimated IC_{50} values against these colon cancer cells are achievable internally. Human diet is complex and the supply of coumaric acid from different diets has to be evaluated simultaneously to have an idea

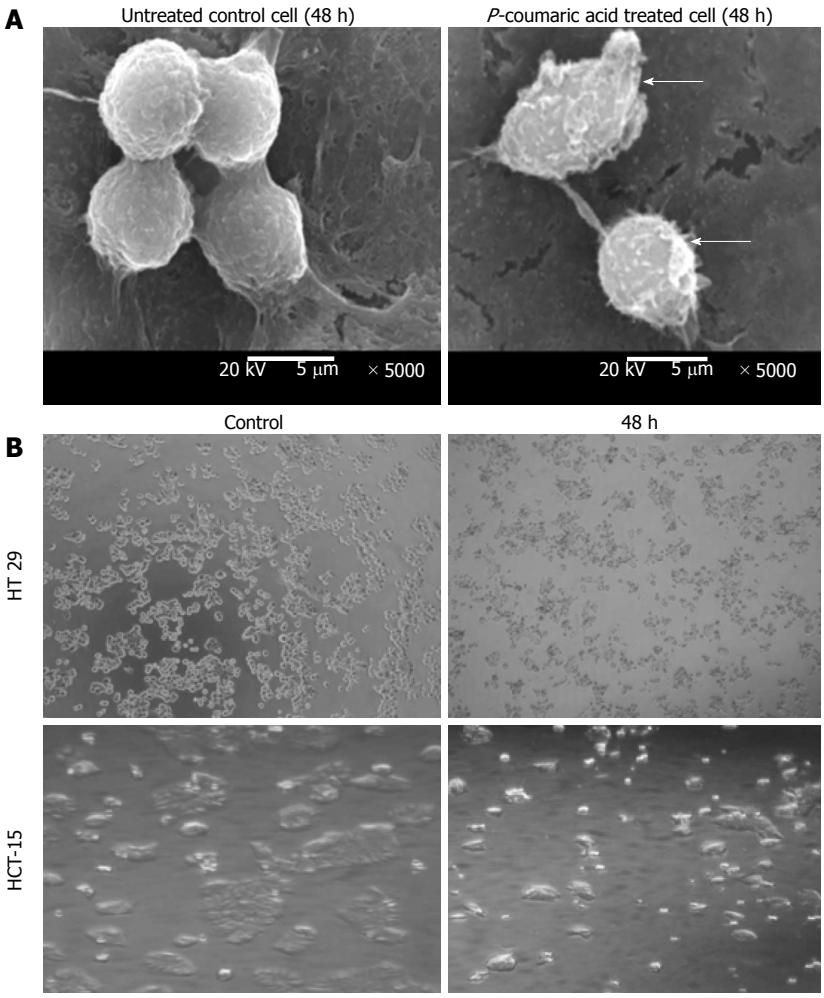


Figure 4 Morphological assessment of *p*-Coumaric acid treated cells. A: Human colorectal carcinoma (HCT-15) cells were treated with *p*-Coumaric acid for 48 h and the cells were observed under scanning electron microscope. Treated cells showed membrane blebbing and shrinkage compared to untreated normal control cells; B: HT 29 and HCT 15 cells were subjected to *p*-Coumaric acid treatment for 48 h and observed under light microscopy. Treated cells displayed apoptotic features like blebbing and shrinkage compared to untreated normal control cells.

about its bioavailability. To add further, bioavailability varies among the individuals and this makes estimation of intakes and prediction of physiological range of phenolics in body fluids is a mammoth task. The biggest drawback is that bioavailability of *p*-Coumaric acid will be in pulses depending upon the food intake whereas in cell culture environments it is constant[10].

p-Coumaric acid significantly inhibited the colony formation *in vitro*. This is indispensable, since most of the chemotherapeutic drugs were shown to inhibit the colony formation[12]. The effect of *p*-coumaric acid against intestinal epithelial cells (IEC) isolated from the mouse was evaluated. It was found that *p*-Coumaric acid was not toxic to these cells. Even at a higher concentration of 5.1 mmol/L nearly 80% cells were viable (results not shown). Sparing nature of *p*-Coumaric acid against mouse IEC was interesting and would warrant further study with normal human colonic cells.

Mitochondrial malfunction is one of the key events occurring at the initial stages of apoptosis. Studies reported a fall in the mitochondrial membrane potential during apoptosis induced by various chemotherapeutic drugs. Mitochondrial membrane potential of *p*-Coumaric acid treated cells using rhodamine-123 showed decreasing intensity, confirming the mitochondrial malfunction. ROS is involved in various biochemical functions like cell proliferation and apoptosis. Generally, ROS stress is oncogenic and it is found to increase the metabolic activity. It also stimulates further ROS generation through mitochondrial respiratory chain and maintains the cancer phenotype. On the other hand, high dose of ROS for prolonged duration could induce cellular damage and apoptosis[19,20]. Hence by utilizing time and dose-dependent ROS generation, we can trigger cell death by using exogenous ROS-generating agents. Our experiment involving DCFDH-DA staining indicated increasing ROS generation in the *p*-Coumaric acid treated cells. Hence, *p*-Coumaric acid may be considered as a potential exogenous candidate (generating ROS) to induce apoptosis in colon cancer cells.

The most notable property of phenolic phytochemicals is that they have antioxidant activity. This is due to

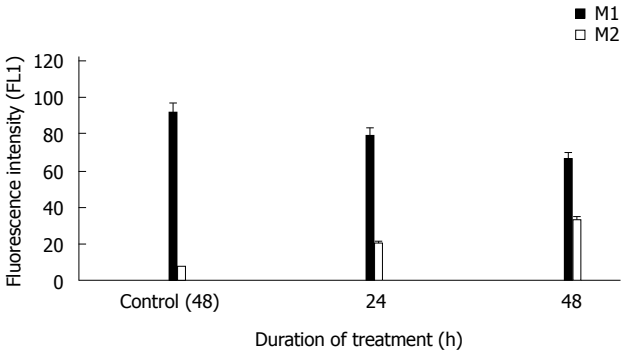


Figure 5 Apoptosis evaluation using Yo-Pro-1 dye by flow cytometry. Human colorectal carcinoma (HCT-15) cells were treated with *p*-Coumaric acid for specified time points. The distribution of cell population changed according to the exposure time as indicated by M1 and M2. Percentage of M2 population depicting apoptosis increased on the basis of the duration of treatment. Data is representative of three independent experiments and the differences in the values of M2 were significant at 24 and 48 h ($P < 0.05$ *vs* untreated control cells) compared to untreated control cells.

the ability of phenolic hydroxyl groups which can provide hydrogen atoms in scavenging the ROS. Hence it is suggested that phenolic phytochemicals could scavenge the ROS molecules and inhibit the mitogen activated protein kinase (MAPK) signaling and blocking the nuclear factor kappaB and activator protein 1 activation which eventually lead to inhibit cancer cell proliferation. Although antioxidant properties of phenolic phytochemicals were explained for its mechanism of inhibiting cancer cells, they also show pro-oxidant activity under certain experimental conditions[21]. ROS generation was observed in the cell culture media containing EGCG, quercetin and gallic acid in both time and concentration-dependent manner[22]. In our case, *p*-Coumaric acid was also found to increase ROS generation in a time-dependent manner. Hence, treating the cancer cells with *p*-Coumaric acid can produce significant ROS resulting stressful or cytotoxic effects. Excess of ROS generation by phenolic phytochemicals induces apoptosis through MAPK activation. Simultaneously, increased p53 activation was mediated by Ras/MAPK kinase/MAPK pathway as observed in the apoptosis of EGCG and reseveratrol[23,24]. Hence, we hypothesize that the increased ROS generation may result in the activation of p53 in the *p*-Coumaric acid treated cells. This may in-turn would have caused the up-regulation of Bax and down-regulation of Bcl2 which are the down-stream targets of p53 resulting in apoptosis.

Apoptosis, a distinguished form of cell death, is characterized by membrane blebbing and DNA fragmentation. Electron Microscopy is used as a golden standard in detecting apoptosis[25-27]. In our case, both scanning electron microscope and photomicrograph images of *p*-Coumaric acid treated cells showed typical membrane blebbing and shrinkage portraying apoptosis. Sub-G_1 arrest of cell cycle is considered as a sign of apoptosis[28-30]. *p*-Coumaric acid treatment showed increasing accumulation of cells in the sub-G_1 phase confirming apoptosis. This was similar to the most anticancer drugs which inducted apoptosis by arresting the cells at sub-G_1 phase[31-33]. At an early stage of apoptosis, there will be considerable damage to plasma membrane and the lipid layer will be disorganized. Nowadays in addition to the nuclear and morphological assessment, lipid layer perturbations in plasma membrane can also insinuate apoptosis. Merocyanine staining of treated cells for lipid layer organization showed increasing fluorescence intensity depicting apoptosis. This observation was similar to eugenol induced apoptosis shown recently[13].

In conclusion, *p*-Coumaric acid exerted antiproliferative activity against colon cancer cells like HT 29 and HCT 15. Both the cell lines growth was repressed significantly by inducing apoptosis. Apoptosis induced by *p*-Coumaric acid involved various physical and biochemical changes. To enumerate, cells showed membrane blebbing and shrinkage as depicted by SEM and photomicrograph images. Earlier lipid layer breaks were associated with the *p*-Coumaric acid induced apoptosis. Cell cycle progression was arrested at sub-G_1 phase by *p*-Coumaric acid treatment. Mitochondrial membrane potential of treated cells also showed a decrease after *p*-Coumaric acid treatment. Moreover, there was increase in the ROS generation and lipid layer breaks after treatment. These results insinuate that *p*-Coumaric acid inhibited the growth of colon cancer cells by inducing apoptosis through ROS-mitochondrial pathway. However, further experiments in preclinical and clinical settings will promote this as a likely candidate for chemoprevention of colon cancer.

ACKNOWLEDGMENTS

Jaganathan SK acknowledges the directors' (Mr. Raguram, Dr. Lakshmana Prabhu, and Mr. Sugumaran) invaluable support and their constant encouragement. Authors acknowledge the efforts of Dr. Joseph Thomas for providing his valuable comments for English-editing service. Thanks to Ms. Bhuvaneswari S and Mr. Joseph Raja for checking this paper.

COMMENTS

Background

Consumption of phenolic components has been linked to improve various disease conditions like cancer, diabetes and cardiac disorders. Diet is believed to be much influential in explaining the susceptibility to cancer. Most interestingly, colon cancer is more vulnerable to diet because these epithelial cells are chronically exposed to these dietary agents. Honey has been reported to possess protective effect in many inflammatory diseases and oxidative stress-related injuries. Recent works from the laboratory showed phenolic components of honey were attributed with inherent potential to inhibit colon cancer cells. In this article *p*-Coumaric acid, one of the phenolic components of honey, has been examined for its growth inhibitory effects.

Research frontiers

Chemotherapy utilizes antineoplastic or dietary agents for treating colon cancer. However, there is still a continuous search for novel agents with improved efficiency. To their knowledge *p*-Coumaric acid, one of the phenolic components of honey, have never been examined for its inhibitory mechanism against colon

cancer.

Innovations and breakthroughs

Events associated with the inhibitory nature of *p*-Coumaric acid are clearly highlighted in this manuscript. Authors have shown that *p*-Coumaric acid inhibited the colon cancer cells in dose-dependent manner. Further it was deciphered that *p*-Coumaric acid induced apoptosis is accompanied with increasing reactive oxygen species (ROS) levels, a fall in the mitochondrial membrane potential and increased lipid layer breaks. Hence authors concluded that *p*-Coumaric acid inhibited the growth of colon cancer cells by inducing apoptosis through ROS-mitochondrial pathway.

Applications

p-Coumaric acid induced apoptosis in colon cancer cells through ROS-mitochondrial pathway. Hence, further experiments in preclinical and clinical settings will promote *p*-Coumaric acid as a plausible candidate for chemoprevention of colon cancer.

Peer review

This work describes the events associated with the growth-inhibitory effect of *p*-Coumaric acid in colon cancer cells. Since *p*-Coumaric acid is one of the phenolic components of honey, the study has a clear interest in the field of chemoprevention of colon cancer. The results of this study are interesting and demonstrate that *p*-Coumaric acid has antiproliferative activity against colon cancer cells inducing apoptosis and causing physical and biochemical changes.

REFERENCES

1 **Yang CS**, Landau JM, Huang MT, Newmark HL. Inhibition of carcinogenesis by dietary polyphenolic compounds. *Annu Rev Nutr* 2001; **21**: 381-406 [PMID: 11375442 DOI: 10.1146/annurev.nutr.21.1.381]

2 **Johnson IT**. Anticarcinogenic effects of diet-related apoptosis in the colorectal mucosa. *Food Chem Toxicol* 2002; **40**: 1171-1178 [PMID: 12067580 DOI: 10.1016/S0278-6915(02)00051-0]

3 **Jaganathan SK**, Mandal M. Honey constituents and its apoptotic effect in colon cancer cells. *JAAS* 2009; **1**: 29-36 [DOI: 10.3896/IBRA.4.01.2.02]

4 **Jaganathan SK**, Mandal SM, Jana SK, Das S, Mandal M. Studies on the phenolic profiling, anti-oxidant and cytotoxic activity of Indian honey: in vitro evaluation. *Nat Prod Res* 2010; **24**: 1295-1306 [PMID: 20803373 DOI: 10.1080/14786410903184408]

5 **Jaganathan SK**, Mondhe D, Wani ZA, Pal HC, Mandal M. Effect of honey and eugenol on Ehrlich ascites and solid carcinoma. *J Biomed Biotechnol* 2010; **2010**: 989163 [PMID: 20369055 DOI: 10.1155/2010/989163]

6 **Ferguson LR**, Lim IF, Pearson AE, Ralph J, Harris PJ. Bacterial antimutagenesis by hydroxycinnamic acids from plant cell walls. *Mutat Res* 2003; **542**: 49-58 [PMID: 14644353 DOI: 10.1016/J.MRGENTOX.2003.08.005]

7 **Kroon PA**, Williamson G. Hydroxycinnamates in plants and food: current and future perspectives. *J Sci Food Agric* 1999; **79**: 355-361 [DOI: 10.1002/(SICI)1097-0010(19990301)79:3<355::AID-JSFA255>3.0.CO;2-G]

8 **Larrosa M**, Lodovici M, Morbidelli L, Dolara P. Hydrocaffeic and p-coumaric acids, natural phenolic compounds, inhibit UV-B damage in WKD human conjunctival cells in vitro and rabbit eye in vivo. *Free Radic Res* 2008; **42**: 903-910 [PMID: 18985489 DOI: 10.1080/10715760802510077]

9 **Abdel-Wahab MH**, El-Mahdy MA, Abd-Ellah MF, Helal GK, Khalifa F, Hamada FM. Influence of p-coumaric acid on doxorubicin-induced oxidative stress in rat's heart. *Pharmacol Res* 2003; **48**: 461-465 [PMID: 12967591]

10 **Janicke B**, Onning G, Oredsson SM. Differential effects of ferulic acid and p-coumaric acid on S phase distribution and length of S phase in the human colonic cell line Caco-2. *J Agric Food Chem* 2005; **53**: 6658-6665 [PMID: 16104781 DOI: 10.1021/jf050489l]

11 **Jaganathan SK**, Mandal M. Involvement of non-protein thiols, mitochondrial dysfunction, reactive oxygen species and p53 in honey-induced apoptosis. *Invest New Drugs* 2010; **28**: 624-633 [PMID: 19705065 DOI: 10.1007/s10637-009-9302-0]

12 **Jaganathan SK**. Growth inhibition by caffeic acid, one of the phenolic constituents of honey, in HCT 15 colon cancer cells. *ScientificWorldJournal* 2012; **2012**: 372345 [PMID: 22649289 DOI: 10.1100/2012/372345]

13 **Jaganathan SK**, Mazumdar A, Mondhe D, Mandal M. Apoptotic effect of eugenol in human colon cancer cell lines. *Cell Biol Int* 2011; **35**: 607-615 [PMID: 21044050 DOI: 10.1042/CBI20100118]

14 **Key TJ**. Fruit and vegetables and cancer risk. *Br J Cancer* 2011; **104**: 6-11 [PMID: 21119663 DOI: 10.1038/sj.bjc.6606032]

15 **Boffetta P**, Couto E, Wichmann J, Ferrari P, Trichopoulos D, Bueno-de-Mesquita HB, van Duijnhoven FJ, Büchner FL, Key T, Boeing H, Nöthlings U, Linseisen J, Gonzalez CA, Overvad K, Nielsen MR, Tjønneland A, Olsen A, Clavel-Chapelon F, Boutron-Ruault MC, Morois S, Lagiou P, Naska A, Benetou V, Kaaks R, Rohrmann S, Panico S, Sieri S, Vineis P, Palli D, van Gils CH, Peeters PH, Lund E, Brustad M, Engeset D, Huerta JM, Rodríguez L, Sánchez MJ, Dorronsoro M, Barricarte A, Hallmans G, Johansson I, Manjer J, Sonestedt E, Allen NE, Bingham S, Khaw KT, Slimani N, Jenab M, Mouw T, Norat T, Riboli E, Trichopoulou A. Fruit and vegetable intake and overall cancer risk in the European Prospective Investigation into Cancer and Nutrition (EPIC). *J Natl Cancer Inst* 2010; **102**: 529-537 [PMID: 20371762 DOI: 10.1093/jnci/djq072]

16 **Wollgast J**, Anklam E. Review on polyphenols in Theobroma cacao: changes in composition during the manufacture of chocolate and methodology for identification and quantification. *Food Res Int* 2000; **33**: 423-447 [DOI: 10.1016/S0963-9969(00)00068-5]

17 **Madhavi DV**, Despande SS, Salunkhe DK. In: Food Antioxidants. New York: Marcel Dekker Publisher, 1996: 267-311

18 **Manach C**, Scalbert A, Morand C, Rémésy C, Jiménez L. Polyphenols: food sources and bioavailability. *Am J Clin Nutr* 2004; **79**: 727-747 [PMID: 15113710]

19 **Trachootham D**, Zhou Y, Zhang H, Demizu Y, Chen Z, Pelicano H, Chiao PJ, Achanta G, Arlinghaus RB, Liu J, Huang P. Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by beta-phenylethyl isothiocyanate. *Cancer Cell* 2006; **10**: 241-252 [PMID: 16959615]

20 **Pelicano H**, Carney D, Huang P. ROS stress in cancer cells and therapeutic implications. *Drug Resist Updat* 2004; **7**: 97-110 [PMID: 15158766]

21 **Loo G**. Redox-sensitive mechanisms of phytochemical-mediated inhibition of cancer cell proliferation (review). *J Nutr Biochem* 2003; **14**: 64-73 [PMID: 12667597 DOI: 10.1016/S0955-2863(02)00251-6]

22 **Long LH**, Clement MV, Halliwell B. Artifacts in cell culture: rapid generation of hydrogen peroxide on addition of (-)-epigallocatechin, (-)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. *Biochem Biophys Res Commun* 2000; **273**: 50-53 [PMID: 10873562 DOI: 10.106/bbrc.2000.2895]

23 **Chen C**, Yu R, Owuor ED, Kong AN. Activation of antioxidant-response element (ARE), mitogen-activated protein kinases (MAPKs) and caspases by major green tea polyphenol components during cell survival and death. *Arch Pharm Res* 2000; **23**: 605-612 [PMID: 11156183 DOI: 10.1007/BF02975249]

24 **Shih A**, Davis FB, Lin HY, Davis PJ. Resveratrol induces apoptosis in thyroid cancer cell lines via a MAPK- and p53-dependent mechanism. *J Clin Endocrinol Metab* 2002; **87**: 1223-1232 [PMID: 11889192 DOI: 10.1007/s00125-012-2747-2]

25 **Taatjes DJ**, Sobel BE, Budd RC. Morphological and cytochemical determination of cell death by apoptosis. *Histochem Cell Biol* 2008; **129**: 33-43 [PMID: 18000678 DOI: 10.1007/s00418-007-0356-9]

26 **Yasuhara S**, Zhu Y, Matsui T, Tipirneni N, Yasuhara Y, Kaneki M, Rosenzweig A, Martyn JA. Comparison of comet assay, electron microscopy, and flow cytometry for detection of apoptosis. *J Histochem Cytochem* 2003; **51**: 873-885 [PMID: 12810838 DOI: 10.1177/002215540305100703]

27 **Martinez MM**, Randall DR, Pappas D. Detection of apop-

tosis: A review of conventional and novel techniques. *Anal Methods* 2010; **2**: 996-1004 [DOI: 10.1039/C0AY00247J]
28 **Sun PC**, Tzao C, Chen BH, Liu CW, Yu CP, Jin JS. Suberoylanilide hydroxamic acid induces apoptosis and sub-G1 arrest of 320 HSR colon cancer cells. *J Biomed Sci* 2010; **17**: 76 [PMID: 20846458 DOI: 10.1186/1423-0127-17-76]
29 **Zheng PW**, Chiang LC, Lin CC. Apigenin induced apoptosis through p53-dependent pathway in human cervical carcinoma cells. *Life Sci* 2005; **76**: 1367-1379 [PMID: 15670616 DOI: 10.1016/j.lfs.2004.08.023]
30 **Chiruvella KK**, Raghavan SC. A natural compound, methyl angolensate, induces mitochondrial pathway of apoptosis in Daudi cells. *Invest New Drugs* 2011; **29**: 583-592 [PMID: 20169399 DOI: 10.1007/s10637-010-9393-7]
31 **Wang YF**, Shyu HW, Chang YC, Tseng WC, Huang YL, Lin KH, Chou MC, Liu HL, Chen CY. Nickel (II)-induced cytotoxicity and apoptosis in human proximal tubule cells through a ROS- and mitochondria-mediated pathway. *Toxicol Appl Pharmacol* 2012; **259**: 177-186 [PMID: 22245127 DOI: 10.1016/j.taap.2011.12.022]
32 **Bartholomeusz C**, Yamasaki F, Saso H, Kurisu K, Hortobagyi GN, Ueno NT. Gemcitabine Overcomes Erlotinib Resistance in EGFR-Overexpressing Cancer Cells through Downregulation of Akt. *J Cancer* 2011; **2**: 435-442 [PMID: 21850211 DOI: 10.7150/jca.2.435]
33 **Kumar VB**, Yuan TC, Liou JW, Yang CJ, Sung PJ, Weng CF. Antroquinonol inhibits NSCLC proliferation by altering PI3K/mTOR proteins and miRNA expression profiles. *Mutat Res* 2011; **707**: 42-52 [PMID: 21185843 DOI: 10.1016/j.mrfmmm.2010.12.009]

P- Reviewers: Cadena MP, De Nardi P
S- Editor: Gou SX **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.v19.i43.7735

World J Gastroenterol 2013 November 21; 19(43): 7735-7742
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Screening of *SLC25A13* mutation in the Thai population

Parith Wongkittichote, Chonlaphat Sukasem, Atsuo Kikuchi, Wichai Aekplakorn, Laran T Jensen, Shigeo Kure, Duangrurdee Wattanasirichaigoon

Parith Wongkittichote, Graduate Program in Molecular Medicine, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
Chonlaphat Sukasem, Division of Pharmacogenomics and Personalized Medicine, Department of Pathology, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand
Atsuo Kikuchi, Shigeo Kure, Department of Pediatrics, Tohoku University Graduate School of Medicine, Sendai 980-8577, Japan
Wichai Aekplakorn, Department of Community Medicine, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand
Laran T Jensen, Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
Duangrurdee Wattanasirichaigoon, Division of Medical Genetics, Department of Pediatrics, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, 10400 Thailand
Author contributions: Wongkittichote P and Sukasem C designed the study, performed experiments, analyzed and interpreted data, drafted and revised the article; Kikuchi A designed the study, performed experiments, revised the article; Aekplakorn W designed the study, analyzed and interpreted data, revised the article; Jensen LT was involved in conception of the study, revised the article; Kure S was involved in the conception and design of the study, analyzed, interpreted data, and revised the article; and Wattanasirichaigoon D was involved in the conception and design of the study, analyzed, interpreted data, drafted and revised the article
Supported by A joint grant from Mahidol University Faculty of Science and Ramathibodi Hospital Faculty of Medicine (Jensen LT and Wattanasirichaigoon D); Mahidol University (Wattanasirichaigoon D: 49/2556); the Pharmacogenomics Project, the collaborative project from the Thailand Center of Excellence for Life Science and Mahidol University to Sukasem C; and the Medical Scholars Program of Mahidol University (Wongkittichote P); a recipient (Wattanasirichaigoon D) of Research Career Development Award, Faculty of Medicine Ramathibodi Hospital
Correspondence to: Duangrurdee Wattanasirichaigoon, MD, Professor of Pediatrics, Division of Medical Genetics, Department of Pediatrics, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand. duangrurdee.wat@mahidol.ac.th
Telephone: +66-2-2012782 Fax: +66-2-2012783
Received: July 9, 2013 Revised: August 29, 2013
Accepted: September 16, 2013
Published online: November 21, 2013

Abstract

AIM: To determine the prevalence of *SLC25A13* mutations in the Thai population.

METHODS: A total of 1537 subjects representing the Thai population were screened for a novel pathologic allele p.Met1? (c.2T > C) and six previously known common *SLC25A13* mutations: [Ⅰ] (c.851_854delGTAT), [Ⅱ] (g.IVS11 + 1G > A), [Ⅲ] (c.1638_1660dup), [Ⅳ] (p.S225X), [Ⅴ] (IVS13 + 1G > A), and [XIX] (g.IVS16ins3kb) using a newly developed TaqMan and established HybProbe assay, respectively. Sanger sequencing was employed for specimens showing an aberrant peak to confirm the targeted mutation as well as the unknown aberrant peaks detected. Frequencies of the mutations identified were compared in each region. Carrier frequency and disease prevalence of citrin deficiency caused by *SCL25A13* mutations were estimated.

RESULTS: p.Met1? was identified in the heterozygous state in 85 individuals, giving a carrier frequency of 1/18, which suggests possible selective advantage of this variant. The question of p.Met1? homozygote lethality remains unanswered which may serve as an explanation as to why this homozygote has yet to be identified in patients/controls even with high allele frequency. The p.Met1? mutation has rarely been studied in populations other than Thai and Chinese; therefore, may have been overlooked. Development of the TaqMan assay in the present study would allow a simple, rapid, and cost-effective method for mass screening. Heterozygous mutations: [XIX] and [Ⅰ] were identified in 17 individuals, giving a carrier rate of 1/90 and a calculated homozygote rate of 1/33000. Two novel variants, g.IVS11 + 17C > G and c.1311C > T, of unknown clinical significance were identified at low frequency.

CONCLUSION: This study highlighted the current underestimation of citrin deficiency and suggests the possible selective advantage of the p.Met1? allele.

Key words: Aspartate-glutamate carrier; Isoform 2; Citrin deficiency; Type Ⅱ citrullinemia; Neonatal intrahepatic cholestasis caused by citrin deficiency; *SLC25A13*

Core tip: Citrin deficiency is underestimated in various populations and the high prevalence of some *SLC25A13* variants possibly contribute to uncharacterized predisposition/protection of certain disorders.

Wongkittichote P, Sukasem C, Kikuchi A, Aekplakorn W, Jensen LT, Kure S, Wattanasirichaigoon D. Screening of *SLC25A13* mutation in the Thai population. *World J Gastroenterol* 2013; 19(43): 7735-7742 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7735.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7735

INTRODUCTION

Citrin deficiency (CD) is a genetic disorder inherited in an autosomal recessive pattern[1]. It is caused by mutations in the *SLC25A13* gene encoding the citrin protein, a mitochondrial aspartate-glutamate carrier, isoform 2 (AGC2) and is expressed mainly in the liver[2-5]. The major function of AGC2 is to export mitochondrial aspartate in exchange for cytosolic glutamate[5] and is involved in several metabolic pathways with major contributions to the urea cycle and malate-aspartate shuttle[1,6,7].

There are three distinct age-dependent clinical phenotypes of CD: the mild phenotype of neonatal intrahepatic cholestasis caused by citrin deficiency [Neonatal Intrahepatic Cholestasis caused by Citrin Deficiency (NICCD), Online Mendelian Inheritance in Man (OMIM): 605814], asymptomatic period or failure to thrive and dyslipidemia in young children, and a fatal phenotype of type Ⅱ citrullinemia [Adult Onset Type Ⅱ Citrullinemia (CTLN2), OMIM: 603471] in older children and adults (11-80 years)[1,8,9]. Misdiagnosed or mistreated CTLN2 patients usually have poor outcomes that often result in death due to hyperammonemic encephalopathy[8].

CD is a panethnic disorder, but is relatively more common among East Asian populations. The carrier prevalence among Japanese and Chinese populations has been reported to be approximately 1/65[10-16]. Despite its high prevalence, clinical features and metabolic profiles are diverse among patients, thus making correct diagnosis difficult[1,17,18]. A definitive diagnosis of CD typically requires DNA sequencing analysis of the *SLC25A13* gene, which is time consuming and costly. Alternative methods such as polymerase chain reaction restriction fragment length polymorphisms, GeneScan and SNaPshot have been adapted to detect mutations in the *SLC25A13* gene[4,13,14,16,18-20]. Recently, Kikuchi *et al*[21], reported a new method for identifying common mutations using a real-time polymerase chain reaction (PCR)-based technique combined with melting curve analysis using HybProbes. This method appears to be a rapid and economical approach that is also suitable for the use with a high-throughput platform[21].

Over 30 *SLC25A13* mutations have been described[13,14,19] and our group has previously shown that mutation [Ⅰ] was the most common (8/10) mutated allele identified among NICCD Thai infants with the remaining identified as mutations [Ⅲ] (c.1638_1660dup) and [XIX] (g.IVS16ins3kb)[16]. In addition, the heterozygous state of a novel p.Met1? (c.2T > C) variant was identified in an infant with idiopathic cholestasis and in 3 out of 100 healthy controls[16]. This variant was also observed in Chinese control individuals with equivalent carrier prevalence[20]. The pathogenic property of p.Met1? has been confirmed in a yeast model of CD and this mutant exhibits loss of citrin function[22]. Here, we conducted a detailed investigation to capture the true prevalence of CD in the Thai population given the small number of Thai patients with CD that have been characterized at the molecular level and with a high prevalence of p.Met1? carriers in an earlier study.

MATERIALS AND METHODS

Subjects

Eligible DNA samples were anonymous specimens previously stored and obtained through the Thai 4th National Health Examination Survey, during August 2008 and March 2009 by the National Health Examination Survey Office, Health System Research Institute. Sample size was calculated based on the estimated carrier prevalence of *SLC25A13* mutations, 1/110, which was derived from a previous patient-based study[16]. Using the Power and Sample Size Calculations Program (version 3.0.43), for alpha error of 10% and power of 90%, the target sample size needed to achieve statistical significance was 669. Even in a worst case scenario (carrier rate of 1/200) with the same alpha error and power of testing, the minimum sample size required was 1337. To offset sample loss due to insufficient DNA quantity or the degradation of DNA extract quality, 15%-20% additional specimens were added to the sample size. A computer-based simple randomization was used to randomly select samples from each of the five regions in Thailand including; Bangkok, Central, Northeastern, Northern and Southern (Figure 1). The number of samples from each region was in accordance to the population distribution reported by the National Statistical Office (www.nso.go.th/). This ensured an avoidance of ascertainment bias of the study population since there may or may not have been the possibility of ethnic differences of the *SLC25A13* variants among subpopulations in Thailand. Moreover, Thais from the Southern region of the country are more ethnically re-

Figure 1 Map of Thailand and geographic distribution.

lated to Malay descendents and those from the Northeastern region are more ethnically related to descendants of Laos and Cambodia. In total, 1569 specimens were received for the study following the approval of Ramathibodi Hospital institutional review board.

Detection of the p.Met1? allele by TaqMan assay

Real-time PCR using hydrolysis probes that were specific to each allele were employed to detect the p.Met1? mutation. Probes and primers were designed according to the *SLC25A13* gene sequence (GenBank accession no. NM_014251). Primer sequences were 5' GTCAGTGGGTCCCGCAGTC 3' and 5' GCACCCCATTTTGCTCCG 3' as a forward and reverse primer, respectively. The probe for detecting the wild-type allele was tagged with 6-FAM at the 5' end, whereas the mutant allele contained VIC. Sequences of the wild-type and mutant probes were 6-FAM 5'AACCGGGGCGAATCATGGCG 3' minor groove binder (MGB) and VIC 5'AACCGGGGCGAATCACGGCG 3' MGB, respectively.

Each real-time PCR reaction contained 0.9 μmol/L of each primer, 0.25 μmol/L of each probe, 20 ng of genomic DNA and 5 μL of TaqMan® Genotyping Master Mix (Applied Biosystem). The thermal profile started with an initial denaturation at 95 ℃ for 10 min followed by 55 amplification cycles at 30 s, and denaturation at 95 ℃. Finally, annealing and extension for 90 s at 62 ℃. The ViiA™ 7 System (Applied Biosystem) was used for detection. Positive samples were confirmed using PCR-*Eag*I restriction digest as previously established[16].

Detection of six common mutations by HybProbe assay

Six common mutations accounting for 91%-100% of the mutant alleles previously reported in Japanese, Chinese, and Thai patients were selected for screening in the present study[14,16,21]. These mutations were: [Ⅰ] (c.851_854delGTAT), [Ⅱ] (g.IVS11+1G>A), [Ⅲ] (c.1638_1660dup), [Ⅳ] (p.S225X), [Ⅴ] (IVS13 + 1G > A) and [XIX] (g.IVS16ins3kb). HybProbe assays were performed using probes and primers for *SLC25A13* previously validated[21]. Real-time PCR was performed using a LightCycler 480 (Roche Applied Science). A 10-μL real-time PCR reaction contained 0.5 μmol/L of each forward and reverse primer (except 0.1 μmol/L of reverse primer in the probe-primer set B), 0.2 μmol/L of each donor and acceptor probe, 20-40 ng of genomic DNA and 5 μL of LightCycler 480 probe Master (Roche Applied Science) or Premix Ex Taq™ (Perfect Real Time) (Takara Bio Company). All positive samples were confirmed using PCR-*Hpy*CH4IV restriction digest for mutation [Ⅰ][16], long-range PCR for mutation [XIX][14], and/or direct sequencing (Research Center, Ramathibodi Hospital). Long range PCR conditions were as follows: 94 ℃ for 5 min; 35 cycles of 98 ℃ for 20 s, 60 ℃ for 30 s, 68 ℃ for 15 min; 72 ℃ for 20 min and 15 ℃ for ∞. It should be noted that conventional nomenclature of mutations of the *SLC25A13* gene has been widely used; therefore, to ensure that the readers would easily grasp the mutation type concept, conventional nomenclature was used alongside standard nomenclature.

Statistical analysis

Fisher's Exact test was used to determine the statistical difference for the frequencies of the mutant alleles identified in different geographic regions. A P value of < 0.05 was considered statistically significant. Analysis of variance to compare sex and age distribution among regions was performed using the SPSS program (version 16.0, SPSS Inc., Chicago, IL, United States).

RESULTS

Complete DNA specimens from 1537 individuals were included in the analysis, 758 males (mean age 47 ± 21 years) and 779 females (mean age 49 ± 21 years), with an overall mean age of 48 ± 21 years. Sex and age distribution were not significantly different in the five different regions, although the mean age of subjects from the Northeastern region was slightly higher than those from the other areas of the country.

High prevalence of p.Met1? allele in the Thai population

The p.Met1? allele was found in 85 individuals giving a 1/18 carrier frequency. This mutation was evenly distributed throughout geographic regions (Table 1). One of these individuals was compound heterozygous with mutation [Ⅰ]; however, since the specimens in our analy-

Table 1 Number of individuals with *SLC25A13* mutations and carrier prevalence in the Thai population according to geographical distribution

Region	Mutation [I] (c.851_854delGTAT)	Mutation XIX (g.IVS16ins3kb)	p.Met1?	Total carriers (p.Met1? not included)	Total carriers (p.Met1? included)
Bangkok (n = 142)	2 (1/71)[1]	2 (1/71)	5 (1/28)[1]	4 (1/36)	8 (1/18)[1]
Central (n = 387)	0	3 (1/129)	17 (1/23)	3 (1/129)	20 (1/19)
Northeastern (n = 498)	4 (1/125)	0	27 (1/18)	4 (1/125)	31 (1/16)
Northern (n = 291)	1 (1/291)	3 (1/97)	21 (1/14)	4 (1/74)	25 (1/12)
Southern (n = 219)	1 (1/219)	1 (1/219)	15 (1/15)	2 (1/110)	17 (1/13)
Total (n = 1537)	8 (1/192)	9 (1/171)	85 (1/18)	17 (1/90)	101 (1/15)
P value[2]	0.18	0.067	0.366	NA	0.413

[1]One individual was found with compound heterozygote of Mutation [I] (c.851_854 delGTAT) and p.Met1?; [2]Statistical analysis using Fischer's Exact test giving P value for comparison of the carrier prevalence identified across the regions. NA: Not available.

sis were anonymous it was impossible to determine the phenotype of this person.

Carrier frequency of six other SLC25A13 mutations

A total of 17 individuals were heterozygotes for mutant alleles. Excluding the p.Met1? variant, 8 mutation [I] and 9 mutation [XIX] (Table 1) were observed, giving a 1/90 carrier rate. Frequencies of mutations [I] and [XIX] identified in each region were not statistically different (P = 0.180 and 0.067, respectively). The four other mutations were not found. Melting point analysis revealed four specimens with heterozygous peaks that were distinct from mutation [II] (IVS11 + 1G > A) (Figure 2A). Sequencing of these specimens revealed a novel single nucleotide polymorphism (SNP), IVS11 + 17C > G. This SNP was located in the anchor-probe binding sequence. Prediction by NNSplice[23] (http://www.fruitfly.org/seq_tools/splice.html) indicated no change in the splice-site score.

Screening of mutation [V] revealed one specimen with an abnormal melting peak (Figure 2B) where direct sequencing revealed an SNP, c.1311C > T, at the binding site of the reporter probe. This novel variant was located at the last base of a codon and at the last base of exon 13, likely resulting in a synonymous change, c.C437C. The calculated donor splice score remained unchanged (0.97) suggesting that this variant is likely to be a rare polymorphism.

DISCUSSION

We have demonstrated a carrier rate of at least 1 in 90, and a homozygote/compound homozygote rate of 1 in 33000 for the known and previously identified *SLC25A13* mutations, excluding the p.Met1? variant among the general Thai population. This number is different, although similar, to the estimated carrier prevalence of 1/110 and homozygotes/compound homozygote rate of 1/48000 that was obtained from the only other available patient-based study[16]. Our findings are also consistent with the prevalence of *SLC25A13* homozygotes/compound heterozygotes of 1/17000 in the Japanese population and the disease frequency of 1/17000-34000 for NICCD, and 1/100000-230000 for CTLN2[8,24,25].

No studies of the p.Met1? variant had been carried out prior to the recent description in affected and unaffected populations of China and Thailand[8,14-16,20]. This mutant allele has a high carrier frequency of 1/18 and a predicted homozygote rate of 1/1300. Based on our analysis, approximately 50000 individuals homozygous for p.Met1? are predicted to be present in the Thai population. Proven deleterious effects of the p.Met1? variant in a yeast model[22] and identification of a compound heterozygote between (paternally inherited) p.Met1? and the (maternally inherited) *SLC25A13* pathologic allele (r.16_212dup) in a Chinese NICCD patient[20] supports the pathogenicity of the p.Met1? allele. When taking the p.Met1? allele into account with the other six previously described common mutations identified in the present study, a very high carrier rate, 1 in 15, and homozygote rate, 1 in 900, is predicted for *SLC25A13* mutations.

Patient- and population-based analyses of the prevalence of the p.Met1? variant in other ethnic backgrounds would help reveal its clinical relevance in CD and other disorder (s), if any. Based on the yeast model, the p.Met1? variant is expected to cause citrin protein production loss. It is also possible that in the p.Met1? variant, an alternative translation initiation site may produce a truncated citrin protein lacking 34 amino acid residues. However, the loss of the 34 *N*-terminal citrin residues causes the mislocalization and impaired function of the aberrant protein[22], indicating that even if this truncated protein was produced, it would not contribute to normal citrin activity. Currently, we have not identified a p.Met1? homozygotic individual and it is possible that p.Met1? may lead to embryonic lethality, similar to homozygotes of the Southeast Asian Ovalocytosis mutant allele in the anion-exchanger 1[26,27]. Several questions regarding the p.Met1? mutation still remain unanswered. Despite its deleterious nature in the yeast model, high prevalence in Thai and Chinese populations from tropical areas, resistance to infectious diseases, and unexplainable unidentified homozygotes in patients even with a high allele frequency, its clinical pathology in humans, ethnic and regional prevalence, selective advantage against infectious diseases, and its lethality warrant further study.

There are two noteworthy limitations of the current study: subject age and the investigation of other

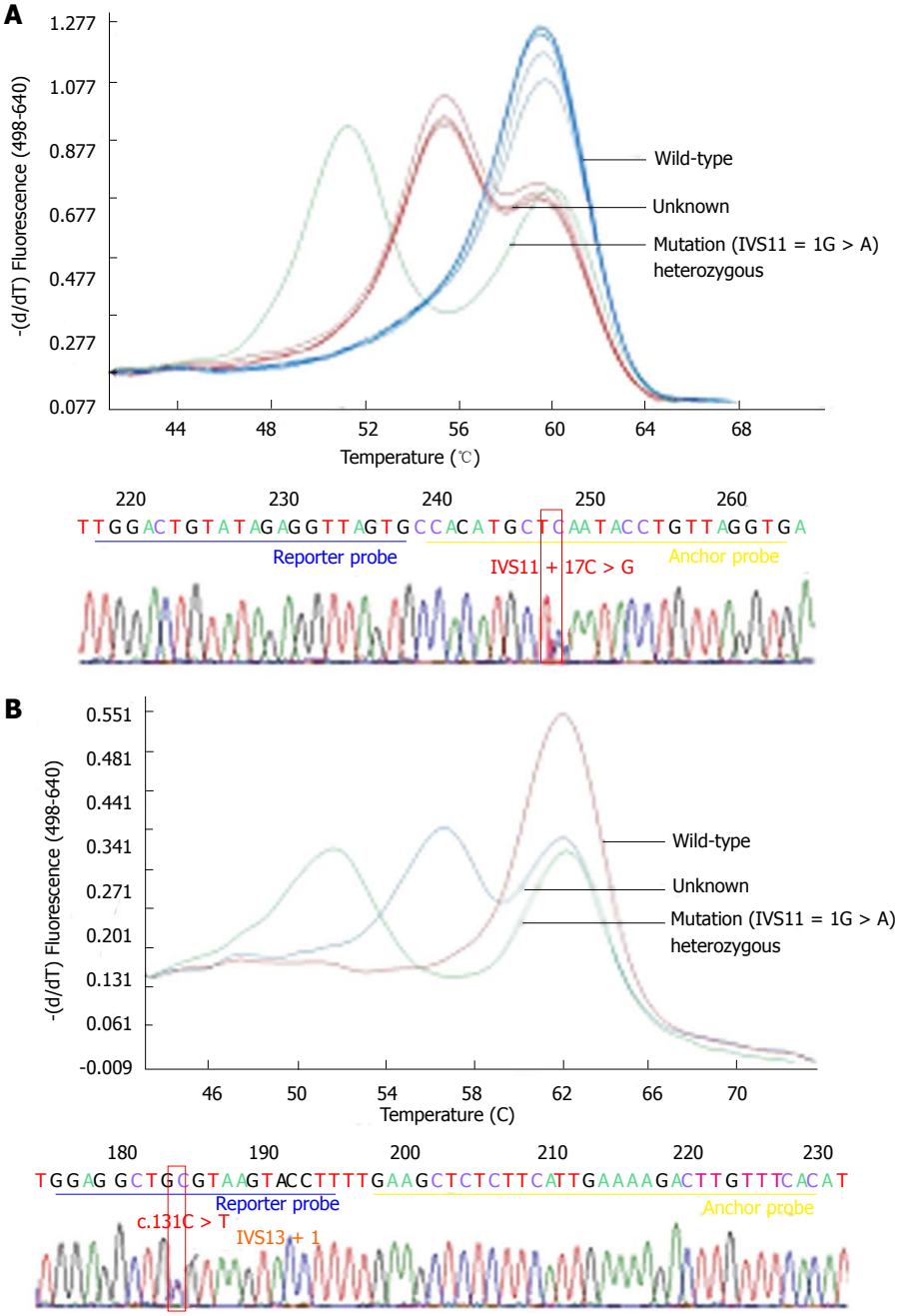


Figure 2 Novel variants in the anchor probe binding sites, detected using the HybProbe assay. A: Upper panel shows unknown heterozygous melting peaks distinguishable from mutation [Ⅱ] (IVS11 + 1G > A) the heterozygous positive control; lower panel is a sequenogram, confirming a variant IVS11 + 17C > G; B: Upper panel shows an aberrant heterozygous melting peak deviated from mutation [Ⅴ] (IVS13 + 1G > A) heterozygote; lower panel indicates a sequenogram, confirming a novel variant c.1311C > T.

mutations. Given that 48 million people in Thailand are represented in our study, based on the inclusion age of 15 or older, over 1455 individuals may possibly have CD considering the confirmed disease causing mutations (excluding p.Met1?). The carrier rate derived from the present study may be underestimated due to the age of the population and mutations screened. Subjects involved in the study were living adults with an average age of 48 years. Some homozygotes/compound heterozygotes might not have survived prior to the start of the survey due to severe phenotypes, and thus would not have been included in our analysis. However, the age of the subjects may not have such a significant effect on the results of this study since the severe phenotype is relatively rare. Another limitation of the present study was that only six mutations were screened, thus the contribution of other mutations was not included in our analysis. The other two less common mutations among the Chinese and Japanese population: [Ⅵ] (c.1799_1800insA) and [Ⅷ] (c.1801G > T)[21] were not selected for screening due to budget limitations.

CD is relatively common in East Asian populations[13,14]. Data from this study suggest that it is also common in Thais. While our analysis did not reveal any significant changes in mutation distribution in the five regions of Thailand, it is possible that an increase in sample size from each region may be necessary to reveal any other variations. CD has also been reported in Vietnamese and Malaysians[14,15,28]. Additional population studies in Southeast Asian populations will shed more light on the geographical distribution of this disease.

When considering each individual confirmed disease

Table 2 Frequency of *SLC25A13* mutations in population studies

Mutation		Number of carriers (allele frequency)				
		Japanese[21]	Japanese[13,14]	Korean[13]	Chinese[13]	Thai (present study)
I	851del4	4 (0.48)	4 (0.15)	11 (0.22)	45 (0.54)	8 (0.26)
II	IVS11 + 1G > A	3 (0.36)	9 (0.33)	8 (0.16)	0 (0.00)	0 (0.00)
III	1638ins23	0 (0.00)	1 (0.04)	1 (0.02)	3 (0.04)	0 (0.00)
IV	S225X	0 (0.00)	5 (0.18)	0 (0.00)	0 (0.00)	0 (0.00)
V	IVS13 + 1G > A	2 (0.24)	1 (0.04)	0 (0.00)	0 (0.00)	0 (0.00)
VII	R605X	0 (0.00)	0 (0.00)	2 (0.04)	0 (0.00)	NA
VIII	E601X	1 (0.12)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
X	IVS6 + 5G > A	0 (0.00)	0 (0.00)	0 (0.00)	15 (0.18)	NA
XI	R184X	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.01)	NA
XIX	IVS16ins3kb	0 (0.00)	1 (0.04)	NA	NA	9 (0.29)
	p.Met1?	NA	NA	NA	NA	85 (2.77)
	n	420	1372	2455	4169	1537
p.Met1? not included						
	n	10	20	22	64	17
	Carrier rate	1/42	1/65	1/112	1/65	1/90
	Homozygote rate	1/7100	1/17000	1/50000	1/17000	1/33000
p.Met1? included						
	n		NA			101
	Carrier rate					1/15
	Homozygote rate					1/900

NA: Not available.

causing mutations, [XIX] and [I] were the two leading mutations identified in the general Thai population with equivalent frequency, 9/1537 and 8/1537, respectively (Table 2), whereas patient-based studies indicate a higher frequency of mutation [I] (8/10 mutated alleles)[16]. The discrepancy between the frequencies of mutant alleles identified from population-based studies and those obtained from patient-based analyses is also evident in other studies[13,21].

Of the known disease causing mutations, [I] and [XIX] are most common in Thai and Chinese populations, whereas mutations [II] and [I] variants are most common among Japanese populations and patients (Table 2)[13,14,21]. In further exploration of this difference in ethnic mutation, when comparing between Chinese and Japanese, there is a possibility that the Thai ethnic background is more closely related to that of the Chinese. This may be linked to the ancient migration of certain Chinese ethnic groups to Thailand[29].

The discovery of g.IVS11 + 17C > G and c.1311C > T variants which are located on the anchor/reporter probe binding sites raised the possibility that an SNP located in the same area may affect the dissociation of probes from the target DNA and interfere with detection of the target SNP. Therefore, direct sequencing should be performed on positive subjects in order to confirm the presence of the target SNP. Moreover, the presence of double SNPs in *cis* could possibly obscure detection of the target SNP; however, this possibility remains to be demonstrated. Bioinformatic analysis of the g.IVS11 + 17C > G and c.1311C > T variants showed no change in splice score or amino acid sequence, suggesting a benign nature of these SNPs. However, the possibility of either being a pathogenic allele cannot be completely excluded due to its surprisingly low prevalence, 4 and 1 in 1537, respectively.

Overall, this study revealed that CD is not uncommon in the Thai population and there is a high frequency of the p.Met1? allele. Once the optimization for TaqMan/HybProbe analysis for each mutation is complete, it will serve as a rapid, efficient, robust, convenient, and cost-saving method for large scale analysis that will enable general population and newborn screening across the country. This has already been demonstrated by the successful establishment of the TaqMan assay for the p.Met1? mutation. Procedures utilized in the present study should prove valuable in examining the distribution and frequencies of *SLC25A13* mutations including p.Met1? among Southeast Asian populations.

Further investigations are required to establish the clinical relevance of p.Met1? both in patients and controls. Demonstration of the molecular pathogenic mechanism of p.Met1? in human/mammalian models, although it is predicted to be a loss of function mutation[22], will also aid in further understanding. The unusually high frequency of the p.Met1? mutation suggests it may have a role in the predisposition and/or protection of disorder (s), perhaps similar to the selection of red cell polymorphisms in areas endemic for malarial infection[30-32]. Our further work will examine the possible connection between the p.Met1? mutation and protection against infectious tropical diseases.

ACKNOWLEDGMENTS

The authors thank Professors Takeyori Saheki and Keiko Kobayashi for their wholehearted support, Dr. Chusak Okascharoen for statistical advice, and Ms. Porawee Kod-

charin for sample preparation assistance.

COMMENTS

Background

Citrin deficiency (CD) leads to three distinct phenotypes; two of which are non-fatal. These phenotypes include: Neonatal intrahepatic cholestasis caused by citrin deficiency, failure-to-thrive and dyslipidemia. The third, a fatal phenotype of type Ⅱ citrullinemia, has marked elevation of ammonia level mimicking a primary disorder of the urea cycle.

Research frontiers

Treatment for CD is distinct from other urea cycle disorders. Epidemiological data are needed to predict disease prevalence and aid in creating awareness for diagnosis among physicians. In this study, the authors screened 1537 subjects from the general population for a novel pathologic allele, p.Met1?, and six common mutations using newly developed Taqman and established HybProbe assays. They demonstrated a carrier frequency of 1/18 for p.Met1? allele, and the carrier rate of 1/90 for mutations: [XIX] and [I], and calculated homozygote rate of 1/33000 for the two latter mutations.

Innovations and breakthroughs

The question of p.Met1? homozygote lethality remains unanswered which may serve as an explanation as to why this homozygote has yet to be identified in patients/controls even with high allele frequency. The p.Met1? mutation has been rarely studied in populations other than the Thai and Chinese and therefore, may have been overlooked. The high carrier rate of p.Met1? suggests the possible selective advantage of this particular allele.

Applications

The unusually high frequency of the p.Met1? mutation suggests its possible role in the predisposition and/or protection of disorder(s), which is perhaps similar to that of the selection of red cell polymorphisms in endemic areas for malarial infection. The established TaqMan assay would allow for a simple, rapid, and cost-effective method for p.Met1? mass screening.

Terminology

CD is caused by a mutation in the *SLC25A13* gene encoding for an aspartate-glutamate carrier isoform 2 (AGC2) and is expressed mainly in the liver. The major function of AGC2 is to export mitochondrial aspartate in exchange for cytosolic glutamate and it is involved in several metabolic pathways with major contributions to the urea cycle and malate-aspartate shuttle.

Peer review

The article covers a topic area of increasing interest. It contains novel information and data affirms on available literature. 1537 subjects from general population were screened for a novel pathologic allele p.Met1? and six common mutations using newly developed Taqman and established HybProbe assays. The study highlighted the current underestimation of citrin deficiency and suggested the possible selective advantage of the p.Met1? allele.

REFERENCES

1 **Saheki T**, Kobayashi K. Mitochondrial aspartate glutamate carrier (citrin) deficiency as the cause of adult-onset type II citrullinemia (CTLN2) and idiopathic neonatal hepatitis (NICCD). *J Hum Genet* 2002; **47**: 333-341 [PMID: 12111366 DOI: 10.1007/s100380200046]

2 **Del Arco A**, Agudo M, Satrústegui J. Characterization of a second member of the subfamily of calcium-binding mitochondrial carriers expressed in human non-excitable tissues. *Biochem J* 2000; **345** Pt 3: 725-732 [PMID: 10642534]

3 **Iijima M**, Jalil A, Begum L, Yasuda T, Yamaguchi N, Xian Li M, Kawada N, Endou H, Kobayashi K, Saheki T. Pathogenesis of adult-onset type II citrullinemia caused by deficiency of citrin, a mitochondrial solute carrier protein: tissue and subcellular localization of citrin. *Adv Enzyme Regul* 2001; **41**: 325-342 [PMID: 11384753 DOI: 10.1016/S0065-2571(00)00022-4]

4 **Kobayashi K**, Sinasac DS, Iijima M, Boright AP, Begum L, Lee JR, Yasuda T, Ikeda S, Hirano R, Terazono H, Crackower MA, Kondo I, Tsui LC, Scherer SW, Saheki T. The gene mutated in adult-onset type II citrullinaemia encodes a putative mitochondrial carrier protein. *Nat Genet* 1999; **22**: 159-163 [PMID: 10369257 DOI: 10.1038/9667]

5 **Palmieri L**, Pardo B, Lasorsa FM, del Arco A, Kobayashi K, Iijima M, Runswick MJ, Walker JE, Saheki T, Satrústegui J, Palmieri F. Citrin and aralar1 are Ca(2+)-stimulated aspartate/glutamate transporters in mitochondria. *EMBO J* 2001; **20**: 5060-5069 [PMID: 11566871 DOI: 10.1093/emboj/20.18.5060]

6 **Begum L**, Jalil MA, Kobayashi K, Iijima M, Li MX, Yasuda T, Horiuchi M, del Arco A, Satrústegui J, Saheki T. Expression of three mitochondrial solute carriers, citrin, aralar1 and ornithine transporter, in relation to urea cycle in mice. *Biochim Biophys Acta* 2002; **1574**: 283-292 [PMID: 11997094 DOI: 10.1016/S0167-4781(01)00376-1]

7 **Saheki T**, Kobayashi K, Iijima M, Moriyama M, Yazaki M, Takei Y, Ikeda S. Metabolic derangements in deficiency of citrin, a liver-type mitochondrial aspartate-glutamate carrier. *Hepatol Res* 2005; **33**: 181-184 [PMID: 16199199 DOI: 10.1016/j.hepres.2005.09.031]

8 **Kobayashi K,** Saheki T, Song Y. Citrin Deficiency Sep 16 [Updated 2012 Jan 5]. In: Pagon RA, Bird TD, Dolan CRea, eds. GeneReviews™ [Internet]. Seattle: University of Washington, Seattle, 1993. Available from: URL: http://www.ncbi.nlm.nih.gov/books/NBK1181/

9 **Ohura T**, Kobayashi K, Tazawa Y, Nishi I, Abukawa D, Sakamoto O, Iinuma K, Saheki T. Neonatal presentation of adult-onset type II citrullinemia. *Hum Genet* 2001; **108**: 87-90 [PMID: 11281457 DOI: 10.1007/s004390000448]

10 **Dimmock D**, Kobayashi K, Iijima M, Tabata A, Wong LJ, Saheki T, Lee B, Scaglia F. Citrin deficiency: a novel cause of failure to thrive that responds to a high-protein, low-carbohydrate diet. *Pediatrics* 2007; **119**: e773-e777 [PMID: 17332192 DOI: 10.1542/peds.2006-1950]

11 **Dimmock D**, Maranda B, Dionisi-Vici C, Wang J, Kleppe S, Fiermonte G, Bai R, Hainline B, Hamosh A, O'Brien WE, Scaglia F, Wong LJ. Citrin deficiency, a perplexing global disorder. *Mol Genet Metab* 2009; **96**: 44-49 [PMID: 19036621 DOI: 10.1016/j.ymgme.2008.10.007]

12 **Fiermonte G**, Parisi G, Martinelli D, De Leonardis F, Torre G, Pierri CL, Saccari A, Lasorsa FM, Vozza A, Palmieri F, Dionisi-Vici C. A new Caucasian case of neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD): a clinical, molecular, and functional study. *Mol Genet Metab* 2011; **104**: 501-506 [PMID: 21914561 DOI: 10.1016/j.ymgme.2011.08.022]

13 **Lu YB**, Kobayashi K, Ushikai M, Tabata A, Iijima M, Li MX, Lei L, Kawabe K, Taura S, Yang Y, Liu TT, Chiang SH, Hsiao KJ, Lau YL, Tsui LC, Lee DH, Saheki T. Frequency and distribution in East Asia of 12 mutations identified in the SLC25A13 gene of Japanese patients with citrin deficiency. *J Hum Genet* 2005; **50**: 338-346 [PMID: 16059747 DOI: 10.1007/s10038-005-0262-8]

14 **Tabata A**, Sheng JS, Ushikai M, Song YZ, Gao HZ, Lu YB, Okumura F, Iijima M, Mutoh K, Kishida S, Saheki T, Kobayashi K. Identification of 13 novel mutations including a retrotransposal insertion in SLC25A13 gene and frequency of 30 mutations found in patients with citrin deficiency. *J Hum Genet* 2008; **53**: 534-545 [PMID: 18392553 DOI: 10.1007/s10038-008-0282-2]

15 **Thong MK**, Boey CC, Sheng JS, Ushikai M, Kobayashi K. Neonatal intrahepatic cholestasis caused by citrin deficiency in two Malaysian siblings: outcome at one year of life. *Singapore Med J* 2010; **51**: e12-e14 [PMID: 20200759]

16 **Treepongkaruna S**, Jitraruch S, Kodcharin P, Charoenpipop D, Suwannarat P, Pienvichit P, Kobayashi K, Wattanasirichaigoon D. Neonatal intrahepatic cholestasis caused by citrin deficiency: prevalence and SLC25A13 mutations among Thai infants. *BMC Gastroenterol* 2012; **12**: 141 [PMID: 23067347 DOI: 10.1186/1471-230X-12-141]

17 **Ohura T**, Kobayashi K, Tazawa Y, Abukawa D, Sakamoto

O, Tsuchiya S, Saheki T. Clinical pictures of 75 patients with neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD). *J Inherit Metab Dis* 2007; **30**: 139-144 [PMID: 17323144 DOI: 10.1007/s10545-007-0506-1]

18 **Yamaguchi N**, Kobayashi K, Yasuda T, Nishi I, Iijima M, Nakagawa M, Osame M, Kondo I, Saheki T. Screening of SLC25A13 mutations in early and late onset patients with citrin deficiency and in the Japanese population: Identification of two novel mutations and establishment of multiple DNA diagnosis methods for nine mutations. *Hum Mutat* 2002; **19**: 122-130 [PMID: 11793471 DOI: 10.1002/humu.10022]

19 **Yasuda T**, Yamaguchi N, Kobayashi K, Nishi I, Horinouchi H, Jalil MA, Li MX, Ushikai M, Iijima M, Kondo I, Saheki T. Identification of two novel mutations in the SLC25A13 gene and detection of seven mutations in 102 patients with adult-onset type II citrullinemia. *Hum Genet* 2000; **107**: 537-545 [PMID: 11153906 DOI: 10.1007/s004390000430]

20 **Zhang ZH**, Lin WX, Deng M, Zhao XJ, Song YZ. Molecular analysis of SLC25A13 gene in human peripheral blood lymphocytes: Marked transcript diversity, and the feasibility of cDNA cloning as a diagnostic tool for citrin deficiency. *Gene* 2012; **511**: 227-234 [PMID: 23022256 DOI: 10.1016/j.gene.2012.09.049]

21 **Kikuchi A**, Arai-Ichinoi N, Sakamoto O, Matsubara Y, Saheki T, Kobayashi K, Ohura T, Kure S. Simple and rapid genetic testing for citrin deficiency by screening 11 prevalent mutations in SLC25A13. *Mol Genet Metab* 2012; **105**: 553-558 [PMID: 22277121 DOI: 10.1016/j.ymgme.2011.12.024]

22 **Wongkittichote P**, Tungpradabkul S, Wattanasirichaigoon D, Jensen LT. Prediction of the functional effect of novel SLC25A13 variants using a S. cerevisiae model of AGC2 deficiency. *J Inherit Metab Dis* 2013; **36**: 821-830 [PMID: 23053473]

23 **Reese MG**, Eeckman FH, Kulp D, Haussler D. Improved splice site detection in Genie. *J Comput Biol* 1997; **4**: 311-323 [PMID: 9278062 DOI: 10.1089/cmb.1997.4.311]

24 **Kobayashi K**, Shaheen N, Kumashiro R, Tanikawa K, O' Brien WE, Beaudet AL, Saheki T. A search for the primary abnormality in adult-onset type II citrullinemia. *Am J Hum Genet* 1993; **53**: 1024-1030 [PMID: 8105687]

25 **Shigematsu Y**, Hirano S, Hata I, Tanaka Y, Sudo M, Sakura N, Tajima T, Yamaguchi S. Newborn mass screening and selective screening using electrospray tandem mass spectrometry in Japan. *J Chromatogr B Analyt Technol Biomed Life Sci* 2002; **776**: 39-48 [PMID: 12127323 DOI: 10.1016/S1570-0232(02)00077-6]

26 **Liu SC**, Jarolim P, Rubin HL, Palek J, Amato D, Hassan K, Zaik M, Sapak P. The homozygous state for the band 3 protein mutation in Southeast Asian Ovalocytosis may be lethal. *Blood* 1994; **84**: 3590-3591 [PMID: 7949112]

27 **Wrong O**, Bruce LJ, Unwin RJ, Toye AM, Tanner MJ. Band 3 mutations, distal renal tubular acidosis, and Southeast Asian ovalocytosis. *Kidney Int* 2002; **62**: 10-19 [PMID: 12081559 DOI: 10.1046/j.1523-1755.2002.00417.x]

28 **Chew HB**, Ngu LH, Zabedah MY, Keng WT, Balasubramaniam S, Hanifah MJ, Kobayashi K. Neonatal intrahepatic cholestasis associated with citrin deficiency (NICCD): a case series of 11 Malaysian patients. *J Inherit Metab Dis* 2010; **33** Suppl 3: S489-S495 [PMID: 21161389 DOI: 10.1007/s10545-010-9248-6]

29 **Baker CJ,** Pasuk P. A history of Thailand. 2th edition; New York: Cambridge University Press, 2009: 1-334

30 **Nagel RL**. Innate resistance to malaria: the intraerythrocytic cycle. *Blood Cells* 1990; **16**: 321-339; discussion 340-349 [PMID: 2257317]

31 **Williams TN**. Human red blood cell polymorphisms and malaria. *Curr Opin Microbiol* 2006; **9**: 388-394 [PMID: 16815736 DOI: 10.1016/j.mib.2006.06.009]

32 **Williams TN**. Red blood cell defects and malaria. *Mol Biochem Parasitol* 2006; **149**: 121-127 [PMID: 16797741 DOI: 10.1016/j.molbiopara.2006.05.007]

P- Reviewers: Guo ZS, Han T, Palmirotta R, Syam AF
S- Editor: Gou SX **L- Editor:** Webster JR **E- Editor:** Wu HL

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v19.i43.7743

World J Gastroenterol 2013 November 21; 19(43): 7743-7750
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Single-incision *vs* three-incision laparoscopic cholecystectomy for complicated and uncomplicated acute cholecystitis

Shu-Hung Chuang, Pai-Hsi Chen, Chih-Ming Chang, Chih-Sheng Lin

Shu-Hung Chuang, Pai-Hsi Chen, Chih-Sheng Lin, Department of Biological Science and Technology, National Chiao Tung University, Hsin-Chu 30068, Taiwan
Shu-Hung Chuang, Pai-Hsi Chen, Department of Surgery, Mackay Memorial Hospital, Hsin-Chu Branch, Hsin-Chu 30071, Taiwan
Chih-Ming Chang, College of Management, Fu Jen Catholic University, New Taipei City 24205, Taiwan
Chih-Ming Chang, Department of Nursing, Mackay Memorial Hospital, Hsin-Chu Branch, Hsin-Chu 30071, Taiwan
Author contributions: Chuang SH designed the study; Chuang SH and Chen PH collected the data; Chuang SH, Chen PH, Chang CM and Lin CS analyzed the data; Chuang SH and Lin CS drafted the manuscript; Lin CS revised the manuscript.
Correspondence to: Chih-Sheng Lin, PhD, Department of Biological Science and Technology, National Chiao Tung University, No. 75 Po-Ai Street, Hsin-Chu 30068, Taiwan. lincs@mail.nctu.edu.tw
Telephone: +886-3-5131338 Fax: +886-3-5729288
Received: June 3, 2013 Revised: August 17, 2013
Accepted: September 15, 2013
Published online: November 21, 2013

Abstract

AIM: To compare the clinical outcome of single-incision laparoscopic cholecystectomy (SILC) and three-incision laparoscopic cholecystectomy (3ILC) for acute cholecystitis.

METHODS: From July 2009 to September 2012, 136 patients underwent SILC or 3ILC for acute cholecystitis at a tertiary referral hospital. One experienced surgeon performed every procedure using 5 or 10 mm 30-degree laparoscopes, straight instruments, and conventional ports. Five patients with perforated gallbladder and diffuse peritonitis and 23 patients with mild acute cholecystitis were excluded. The remaining 108 patients were divided into complicated and uncomplicated groups according to pathologic findings. Patient demography, clinical data, operative results and complications were recorded and analyzed.

RESULTS: Fifty patients with gangrenous cholecystitis, gallbladder empyema, or hydrops were classified as the complicated group, and 58 patients with acute cholecystitis were classified as the uncomplicated group. Twenty-three (46.0%) of the patients in the complicated group (n = 50) and 39 (67.2%) of the patients in the uncomplicated group (n = 58) underwent SILC; all others underwent 3ILC. The postoperative length of hospital stay (PLOS) was significantly shorter in the SILC subgroups than the 3ILC subgroups (3.5 ± 1.1 d *vs* 4.6 ± 1.3 d, $P < 0.01$ in the complicated group; 2.9 ± 1.1 d *vs* 3.7 ± 1.4 d, $P < 0.05$ in the uncomplicated group). The maximum body temperature recorded at day 1 and at day 2 following the procedure was lower in the SILC subgroups, but the difference reached statistical significance only in the uncomplicated group (37.41 ± 0.56 ℃ *vs* 37.80 ± 0.72 ℃, $P < 0.05$ on postoperative day 1; 37.10 ± 0.43 ℃ *vs* 37.57 ± 0.54 ℃, $P < 0.01$ on postoperative day 2). The operative time, estimated blood loss, postoperative narcotic use, total length of hospital stay, conversion rates, and complication rates were similar in both SILC and 3ILC subgroups. The complicated group had longer operative time (122.2 ± 35.0 min *vs* 106.6 ± 43.6 min, $P < 0.05$), longer PLOS (4.1 ± 1.3 d *vs* 3.2 ± 1.2 d, $P < 0.001$), and higher conversion rates (36.0% *vs* 19.0%, $P < 0.05$) compared with the uncomplicated group.

CONCLUSION: SILC is safe and efficacious for patients with acute cholecystitis. The main benefit is a faster recovery than that achieved with 3ILC.

Key words: Single-incision laparoscopic cholecystectomy; Single-incision laparoscopic surgery; Laparoen-

doscopic single site surgery; Cholecystectomy; Acute cholecystitis; Complicated cholecystitis; Gangrenous cholecystitis

Core tip: single-incision laparoscopic cholecystectomy (SILC) is an alternative treatment for uncomplicated benign gallbladder diseases, but its role in acute cholecystitis remains unclear. This comparative analysis of SILC with three-incision laparoscopic cholecystectomy for treating acute cholecystitis represents the largest series to date and proportion of gangrenous cholecystitis patients (30.6%). The well-known drawbacks of SILC - longer operative time and higher cost - were alleviated by the larger paraumbilical incisions facilitating extraction of inflamed gallbladders and reliance on conventional instruments only. The low procedure conversion rate observed for SILC indicated its safety and efficacy for treating acute cholecystitis. SILC providing a faster recovery time was the main benefit to these patients.

Chuang SH, Chen PH, Chang CM, Lin CS. Single-incision *vs* three-incision laparoscopic cholecystectomy for complicated and uncomplicated acute cholecystitis. *World J Gastroenterol* 2013; 19(43): 7743-7750 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7743.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7743

INTRODUCTION

Single-incision laparoscopic cholecystectomy (SILC) is a novel technique comparable to traditional multi-incision laparoscopic cholecystectomy (LC) for uncomplicated benign gallbladder diseases in respect of safety and efficacy[1-3]. In addition to well-established cosmetic advantage; decreased post-operative pain and faster recovery are potential benefits[4-6]. However, higher complication rates in SILC have been reported[7-9]. Therefore, application of this technique in cases of acute cholecystitis should be done with caution[10]. Published SILC studies contain a small number of patients with acute cholecystitis[11-15], while reports comparing SILC and traditional LC for acute cholecystitis are very rare[11].

SILC was developed as a step-by-step evolution of three-incision laparoscopic cholecystectomy (3ILC) and two-incision laparoscopic cholecystectomy (2ILC) in March 2010[16]. Importantly, only conventional instruments were used. Initially, this procedure was only adopted in patients with simple benign gallbladder disease. Since May 2011, however, SILC has been offered as an optional procedure for acute cholecystitis by our clinical practice. This study compares the clinical outcomes following SILC and 3ILC for acute cholecystitis over a period of 39 mo.

MATERIALS AND METHODS

From July 2009 to September 2012, 136 consecutive patients with acute cholecystitis underwent cholecystectomy by a single surgeon at a tertiary referral hospital in Hsin-Chu city, Taiwan. Five patients had perforated gallbladder and diffuse peritonitis and were excluded from the analysis. The role of laparoscopic operation in patients with perforated gallbladders and diffuse peritonitis remains controversial[17-19]. Twenty-three patients with "mild acute cholecystitis" were also excluded. The clinical course of this disease is similar to that of a biliary colic. To eliminate the bias related to disease severity, the enrolled 108 patients were divided into complicated and uncomplicated groups, according to operative and pathologic findings. Gangrenous cholecystitis, gallbladder empyema or hydrops were defined as complicated cholecystitis, while all other findings were defined as uncomplicated cholecystitis.

Patient demography, clinical data, operative results and complications were recorded. A modified APACHE Ⅱ was used as the preoperative prognostic score, namely, low risk: < 5 points, intermediate risk: 6-9 points, and high risk: ⩾ 10 points[20]. The operative time was defined as the interval from initial skin incision to skin closure. Postoperative narcotic use was recorded as the intramuscular pethidine dose (mg) per kilogram of patient body weight (*i.e.*, 1 mg/kg). The postoperative length of hospital stay (PLOS) was defined as the duration between the day of surgery and the day of discharge in the same hospitalization. The total length of hospital stay referred to the total hospitalization duration including readmission for late-onset complications. The maximum body temperature (BT; ℃) of each day was recorded from postoperative day 1 to day 4 for patients who were still hospitalized. Any procedure that failed to be fulfilled as scheduled was regarded as converted. The complications were recorded according to the five-grade Clavien-Dindo classification system[21].

Surgical technique

The details of the surgical techniques have been described previously[16]. In SILC, two 5 mm straight instruments and a 5 mm 30-degree rigid laparoscope were inserted into the abdominal cavity *via* three 5 mm ports in a vertical line at a 2 cm paraumbilical incision on the left side. An optional 2 mm right subcostal incision was made for the passage of a transcystic duct catheter to perform an intraoperative cholangiography (IOC). An assistant controlled the retraction grasper in the middle port. The operator controlled the working instrument in the upper port with the right hand and the laparoscope in the lower port with the left hand (self-camera technique). At the end of the procedure, the lower 5 mm port was upgraded to a 10 mm reusable port for specimen extraction into a retrieval bag. All the fascial defects and the skin incision

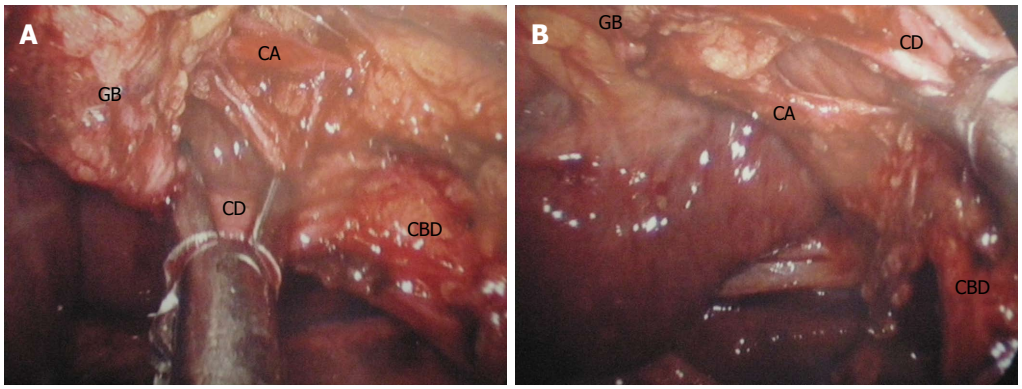


Figure 1 Critical view of safety during a single-incision laparoscopic cholecystectomy for acute cholecystitis. Anterior (A) and posterior (B) views are shown. GB: Gallbladder; CA: Cystic artery; CD: Cystic duct; CBD: Common bile duct.

were sutured. In 3ILC, a 10 mm 30-degree rigid laparoscope was inserted *via* a 10 mm reusable port at a 1 cm infraumbilical incision. The 5 mm working instrument and retraction grasper were inserted *via* two separate 5 mm ports at the epigastrium and right flank respectively.

When a severely inflamed gallbladder or dense pericholecystic fibrosis was encountered at an early stage of the procedure, the threshold for using additional port sites was low. A suction irrigation device was used for decompression of a severely distended gallbladder and meticulous dissection in an unclear operative field. Every effort was made to obtain the critical view of safety (Figure 1), following the recommendations by Strasberg *et al*[22]. If the anatomy of the Calot's triangle was obscure, dissection would be started from the gallbladder dome (retrograde cholecystectomy). In difficult situations, the gallbladder neck or posterior wall was not disturbed (subtotal cholecystectomy)[23,24]. The cystic duct stump or gallbladder neck was secured with intracorporeal suturing if the diameter was too big or the tissue was too fragile to be clipped. In infrequent cases, the liver bed was packed with gauze temporarily if the monopolar electrocautery had failed to achieve hemostasis. Wound extension to fit a firm and swollen gallbladder was usually carried out at the infraumbilical incision in 3ILC, but it was largely unnecessary in SILC. When a subhepatic drain was placed, it was always removed within 48 h after the operation if there was no bile leakage. After discharge, all the patients attended follow-up periods of more than 1 mo.

Statistical analysis

Data were analyzed using Pearson's χ^2 test and Student's *t* test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

The complicated group (gangrenous cholecystitis, gallbladder empyema or hydrops) consisted of 50 patients, and the uncomplicated group (acute cholecystitis) consisted of 58 patients. Twenty-three (46.0%) of the patients in the complicated group and 39 (67.2%) of the patients in the uncomplicated group underwent SILC; the remaining patients all underwent 3ILC. The demographic characteristics, clinical data, and pathologic findings showed no statistically significant differences between the SILC and 3ILC subgroups in either the complicated or uncomplicated groups (Table 1). Patients with gangrenous cholecystitis constituted a major portion of the complicated group (65.2% in the SILC subgroup and 66.7% in the 3ILC subgroup). Preoperative endoscopic retrograde cholangiopancreatography (ERCP) was performed on 13 patients during the same hospitalization to address suspicious concomitant choledocholithiasis (Table 2). Nine of the patients showed a positive result and subsequently underwent immediate therapeutic endoscopic sphincterotomy (EST) for stone clearance. Eleven patients had suspicious concomitant choledocholithiasis without preoperative ERCP and subsequently underwent IOC; the results for all were negative. In cases of positive IOC, common bile duct exploration was performed under laparoscopy.

PLOS and postoperative BT were the only two parameters that displayed a statistical difference between the two subgroups (Tables 2 and 3). The SILC subgroup had a shorter PLOS than the 3ILC subgroup in the complicated and uncomplicated groups ($P < 0.01$ and < 0.05, respectively). The SILC subgroups had a lower maximum BT than the 3ILC subgroups on the postoperative day 1 and day 2 (Figure 2), but the difference reached statistical significance only in the uncomplicated group ($P < 0.05$ for postoperative day 1 and $P < 0.01$ for postoperative day 2). Additional port sites were needed to fulfill the operations in eighteen patients of the complicated group and eleven patients of the uncomplicated group. Converted to an open cholecystectomy (OC) was not necessary in any case. The conversion rates in the SILC and 3ILC subgroups were similar (34.8% *vs* 37.0% in the complicated group; 17.9% *vs* 21.1% in the uncomplicated group).

Fourteen complications occurred in 11 patients (Table 4). The differences in complication rates between the SILC and 3ILC subgroups were statistically insignificant. Four patients experienced mild pulmonary effusion and/or atelectasis, a grade I complication that resolved spon-

Table 1 Patient characteristics and pathology *n* (%)

	Complicated acute cholecystitis		*P* value	Uncomplicated acute cholecystitis		*P* value
	SILC group (*n* = 23)	3ILC group (*n* = 27)		SILC group (*n* = 39)	3ILC group (*n* = 19)	
Age (yr)	51.2 ± 15.3	58.0 ± 17.3	0.147	49.1 ± 13.9	54.6 ± 14.5	0.167
Sex (male/female)	7/16	10/17	0.623	24/15	9/10	0.306
Body mass index (kg/m^2)	25.24 ± 3.36	26.36 ± 3.59	0.272	25.01 ± 2.67	26.99 ± 4.35	0.081
Modified APACHE Ⅱ score (points)			0.318			0.595
0-5, low risk	21 (91.3)	22 (81.5)		36 (92.3)	17 (89.5)	
6-9, intermediate risk	2 (8.7)	5 (18.5)		2 (5.1)	2 (10.5)	
10-11, high risk	0	0		1 (2.6)	0	
Previous abdominal surgery	7	3	0.089	6	5	0.319
Previous biliary symptoms	12	10	0.283	30	11	0.135
Duration of acute symptoms > 72 h	14	14	0.522	34	13	0.087
Pathology			0.985			
Gangrene	15 (65.2)	18 (66.7)		-	-	
Empyema	6 (26.1)	7 (25.9)		-	-	
Hydrops	2 (8.7)	2 (7.4)		-	-	
Acute inflammation	-	-		39 (100)	19 (100)	

SILC: Single-incision laparoscopic cholecystectomy; 3ILC: Three-incision laparoscopic cholecystectomy.

Table 2 Operative modifications and results *n* (%)

	Complicated acute cholecystitis		*P* value	Uncomplicated acute cholecystitis		*P* value
	SILC group (*n* = 23)	3ILC group (*n* = 27)		SILC group (*n* = 39)	3ILC group (*n* = 19)	
Preoperative ERCP	0	3	0.099	0	1	0.148
Preoperative ERCP and EST	2	0	0.118	3	4	0.143
Intraoperative cholangiography	1	0	0.274	5	5	0.202
Operative time (min)	119.8 ± 38.8	124.3 ± 32.1	0.660	100.9 ± 42.1	118.4 ± 45.5	0.154
Estimated blood loss (mL)	43.2 ± 29.8	31.0 ± 26.6	0.156	24.2 ± 31.5	29.5 ± 29.9	0.548
Pethidine dose (mg/kg)	0.624 ± 0.505	0.535 ± 0.740	0.632	0.618 ± 0.485	0.549 ± 0.427	0.601
Postoperative length of hospital stay (d)	3.5 ± 1.1	4.6 ± 1.3	< 0.010	2.9 ± 1.1	3.7 ± 1.4	< 0.050
Total length of hospital stay (d)	6.0 ± 3.6	5.8 ± 3.1	0.814	4.1 ± 1.9	6.4 ± 4.7	0.053
Conversion						
Overall	8 (34.8)	10 (37.0)	0.869	7 (17.9)	4 (21.1)	0.777
2ILC	4	-		3	-	
3ILC	2	-		1	-	
4ILC (standard LC)	2	10		3	4	
OC	0	0		0	0	

SILC: Single-incision laparoscopic cholecystectomy; 2ILC: Two-incision laparoscopic cholecystectomy; 3ILC: Three-incision laparoscopic cholecystectomy; 4ILC: Four-incision laparoscopic cholecystectomy; OC: Open cholecystectomy; ERCP: Endoscopic retrograde cholangiopancreatography; EST: Endoscopic sphincterotomy.

Table 3 Postoperative body temperature

Maximum body temperature (℃)	Complicated acute cholecystitis		*P* value	Uncomplicated acute cholecystitis		*P* value
	SILC group	3ILC group		SILC group	3ILC group	
Post-op day 1	37.73 ± 0.57	38.03 ± 0.66	0.096	37.41 ± 0.56	37.80 ± 0.72	< 0.050
Post-op day 2	37.38 ± 0.59	37.58 ± 0.46	0.180	37.10 ± 0.43	37.57 ± 0.54	< 0.010
Post-op day 3	37.12 ± 0.49	37.30 ± 0.49	0.215	37.04 ± 0.45	37.04 ± 0.56	0.990
Post-op day 4	37.18 ± 0.44	37.19 ± 0.45	0.974	36.88 ± 0.41	36.86 ± 0.33	0.915

Including only patients who were still hospitalized. SILC: Single-incision laparoscopic cholecystectomy; 3ILC: Three-incision laparoscopic cholecystectomy; post-op: Postoperative.

taneously within a few days[21]. Three patients developed grade Ⅱ complications, including infected nonbilious subhepatic collection (*n* = 1), relapsed cholangitis with bacteremia (*n* = 1), and refractory diarrhea (*n* = 1); all were treated conservatively with intravenous antibiotics and fluid therapy. Three patients had grade Ⅲa complications, including infected nonbilious subhepatic collections (*n* = 2) and retained bile duct stones (*n* = 1); the first two patients were managed with percutaneous pigtail drainage and intravenous antibiotics, and the last underwent an ERCP with EST. One patient underwent a laparotomy to remove retained impacted bile duct stones, a grade Ⅲb

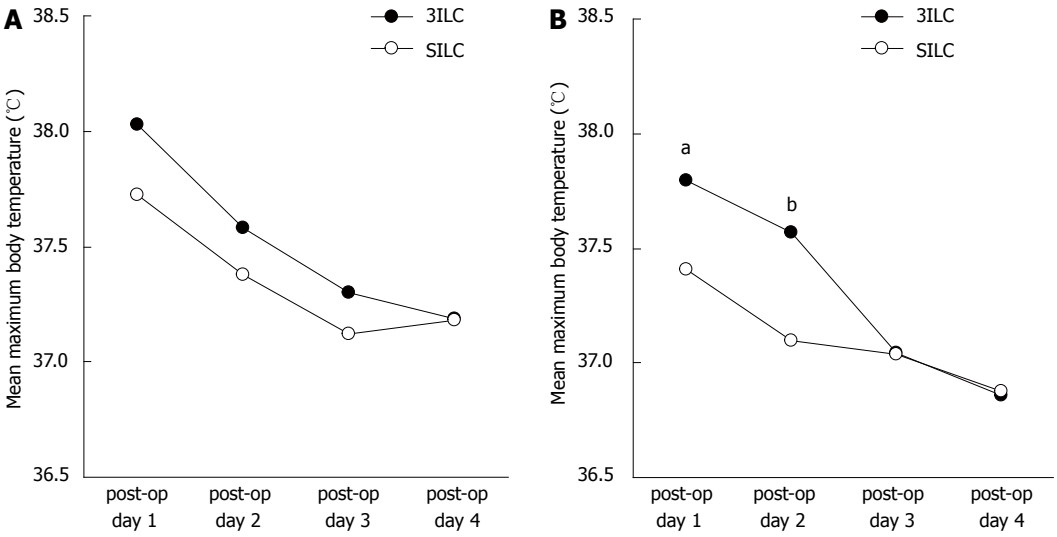


Figure 2 Postoperative mean maximum body temperature for the complicated acute cholecystitis group (A) and the uncomplicated acute cholecystitis group (B). [a]*P* < 0.05, [b]*P* < 0.01 *vs* SILC. 3ILC: Three-incision laparoscopic cholecystectomy; SILC: Single-incision laparoscopic cholecystectomy; post-op: Postoperative.

Table 4 Complications *n* (%)

Complications	Complicated acute cholecystitis		*P* value	Uncomplicated acute cholecystitis		*P* value
	SILC group (*n* = 23)	3ILC group (*n* = 27)		SILC group (*n* = 39)	3ILC group (*n* = 19)	
Overall[1]	3 (13.0)	3 (11.1)	0.834	3 (7.7)	2 (10.5)	0.718
Grade Ⅰ	1 (4.3)[1]	1 (3.7)[2]		2 (5.1)[3]	0	
Grade Ⅱ	1 (4.3)[4]	0		1 (2.6)[5]	1 (5.3)[6]	
Grade Ⅲa	1 (4.3)[7]	2 (7.4)[8]		0	0	
Grade Ⅲb	0	0		0	1 (5.3)[9]	
Grade Ⅳa, Ⅳb, Ⅴ	0	0		0	0	

[1]Pleural effusion and atelectasis; [2]Pleural effusion; [3]One was pleural effusion and atelectasis; the other was pleural effusion; [4]Refractory diarrhea; [5]Infected subhepatic collection; [6]Relapsed cholangitis with bacteremia; [7]Infected subhepatic collection and pleural effusion; [8]One was infected subhepatic collection; the other was retained bile duct stones; [9]Retained bile duct stones. SILC: Single-incision laparoscopic cholecystectomy; 3ILC: Three-incision laparoscopic cholecystectomy.

Table 5 Overall comparison of complicated and uncomplicated acute cholecystitis groups

	Complicated acute cholecystitis (*n* = 50)	Uncomplicated acute cholecystitis (*n* = 58)	*P* value
Age (yr)	54.9 ± 16.6	50.9 ± 14.2	0.184
Sex (male/female)	33/17	33/25	0.333
Modified APACHE Ⅱ score			0.320
0-5, low risk	43 (86.0)	53 (91.4)	
6-9, intermediate risk	7 (14.0)	4 (6.9)	
10-11, high risk	0	1 (1.7)	
Operative time (min)	122.2 ± 35.0	106.6 ± 43.6	< 0.050
Estimated blood loss (mL)	36.1 ± 28.3	26.0 ± 30.8	0.092
Pethidine dose (mg/kg)	0.577 ± 0.633	0.595 ± 0.464	0.867
Postoperative length of hospital stay (d)	4.1 ± 1.3	3.2 ± 1.2	< 0.001
Total length of hospital stay (d)	5.9 ± 3.3	4.8 ± 3.3	0.098
Conversion	18 (36.0)	11 (19.0)	< 0.050
Complications	6 (12.0)	5 (8.6)	0.563

Data are expressed as absolute numbers (percentage) or mean ± SD.

complication. The seven patients with grade Ⅱ, Ⅲa and Ⅲb complications all needed a secondary hospitalization (range: 5-16 d) and recovered uneventfully.

In summary, the complicated group experienced longer operative times ($P < 0.05$), longer PLOS ($P < 0.001$), and higher conversion rates ($P < 0.05$) (Table 5).

DISCUSSION

SILC, also known as laparoendoscopic single site cholecystectomy, has increased in popularity worldwide in recent years. While multiple studies have reported this novel technique to be as safe as traditional LC for the

treatment of uncomplicated benign gallbladder disease[1-3], some have demonstrated that SILC is associated with a higher complication rate[7-9]. Applying SILC in more complex circumstances, such as acute cholecystitis, becomes an interesting angle in which to study SILC in complex circumstances. To date, the published SILC studies have focused on only a small number of patients with acute cholecystitis and comparative studies have been rare[11-15].

According to the 2010 Society of American Gastrointestinal and Endoscopic Surgeons guideline for the clinical application of laparoscopic biliary tract surgery, the indications, contra-indications and preoperative preparation for SILC are the same as those for multi-port cholecystectomy[17]. Both procedures should share the same safety standards with a low conversion threshold. We strictly followed these safety guidelines. Before adopting this technique for acute cholecystitis in May 2011, we had performed over 50 complication-free SILC procedures for simple benign gallbladder disease. Additionally, we are proficient at modified techniques to manage gallbladder complications, such as decompression, meticulous dissection with a suction irrigation device, retrograde cholecystectomy, subtotal cholecystectomy, and intracorporeal suturing the cystic duct stump or gallbladder neck. In SILC, a subhepatic drain always passed through an additional port site. Firm, fragile or severely inflamed gallbladders are usually difficult to retract. Therefore, conventional straight instruments were used in our cases, as the more elastic nature of curved or angulated instruments are not suitable. Because we only used conventional instruments, the procedures could be easily and rapidly converted to multi-incision laparoscopic or open operations for safety concerns.

Pathologic findings often have an effect on operative results of LC[25-27]. To eliminate this bias, we divided patients into two groups according to disease severity. The comparison between complicated and uncomplicated groups showed significant differences in operative time ($P < 0.05$), PLOS ($P < 0.001$), and conversion rates ($P < 0.05$) (Table 5). The findings implicated that the two groups were different. The difference in complication rates did not reach statistical significance. This may be due to inadequate patient number and low complication rates.

The finding that the SILC subgroups had a shorter PLOS than the 3ILC subgroups was consistent with our previous study (Table 2)[16]. Even small traumatic effects can influence postoperative recovery. In case of acute cholecystitis, we followed a rule that patients who tolerated oral feeding well and had a BT under 37.5 ℃ for more than 24 h should be discharged. In this study, all the patients resumed oral feeding the morning after the operation, and most of them tolerated it well. Accordingly, postoperative fever became the critical factor leading to longer PLOS. The finding that the SILC subgroups had a lower maximum BT than the 3ILC subgroups on the postoperative day 1 and day 2 explained the shorter PLOS in the SILC subgroups (Table 3, Figure 2). Although the difference in maximum BT reached statistical significance only in the uncomplicated group, we were more concerned about the postoperative fever in patients with complicated acute cholecystitis. We tended to associate the febrile episodes with postoperative infection in these patients. Accordingly, the small difference in maximum postoperative BT between the SILC and 3ILC subgroups in the complicated group influenced the PLOS significantly. The occurrence of postoperative fever was related to the inflammatory response to cholecystitis, atelectasis, and postoperative septic sequelae. Considering the similar pathologic distributions (disease severity) and postoperative complication rates in the SILC and 3ILC subgroups, it is possible that atelectasis may account for the difference in postoperative BT. Upper abdominal incision (upper midline or subcostal incisions) is a well-established risk factor for the development of atelectasis after abdominal surgery[28], and traditional multi-incision LC was associated with impaired postoperative pulmonary function and an incidence of atelectasis up to 30% in several studies[29-31]. A lower incidence of febrile episodes following LC correlated with improved postoperative pulmonary function and minimal surgical trauma was observed[32]. Thus, 3ILC caused more febrile episodes in the first two postoperative days, and small upper abdominal incisions played a role in impaired postoperative pulmonary function (atelectasis) for patients with acute cholecystitis. We hypothesize that the faster recovery following SILC may be derived not from decreased pain severity, but rather location.

The operative duration and pethidine dose showed no significant difference between the two subgroups (Table 2). It is our opinion that the longer SILC duration in our previous study simply reflected an effect of the learning curve[16,33,34]. The typical SILC procedure for simple benign gallbladder disease takes less than one hour. In addition, the 1 cm infraumbilical incision in a 3ILC was too small to fit a swollen gallbladder, and it took some time to enlarge the incision during specimen extraction. The operative duration spent in SILC and 3ILC was comparable in most cases, for both simple and complicated gallbladder disease. Although we failed to reveal the difference in postoperative narcotic use between the two subgroups, it is too early to make a conclusion. In our clinical practice, a steady intramuscular pethidine dose is available, but the patients may feel pain in different degrees. To clarify the issue of postoperative pain related to the procedures, more detailed studies are necessary.

The outcome following a converted LC is worse than that following a successful LC[35,36]. A qualified laparoscopic surgeon should never hesitate to convert the procedure in an early stage if patient safety is questionable in difficult situations. The high conversion rate in our study (36% in the complicated group and 19% in the uncomplicated group) reflected our safety concerns (Table 5). Consistent with other studies, no procedure was converted to an OC[23,24]. The above-mentioned modified laparoscopic procedures for severe cholecystitis, such as

gallbladder decompression, dissection with a suction irrigation device, retrograde cholecystectomy, subtotal cholecystectomy and intracorporeal suturing, might reduce the open conversion rates tremendously without increasing the complication rates.

In conclusion, SILC with conventional instruments is as safe and efficacious as traditional multi-incision LC for both complicated and uncomplicated acute cholecystitis in experienced hands. The complication rate is low, and the major benefit for patients is faster postoperative recovery. Before applying SILC in difficult gallbladders, a surgeon must be proficient in this novel technique for simple gallbladder disease and the modified laparoscopic techniques for severe cholecystitis. A low threshold for converting the procedure should be maintained for patient safety. Further prospective randomized trials are needed to verify our findings.

ACKNOWLEDGMENTS

We gratefully acknowledge the work of Ms. Yi-Chun Liao, Ms. Chia-Lin Wu and Ms. Jyun-Ya Li in assisting with the data collection. This study was presented at the 21st International Congress of the European Association for Endoscopic Surgery in Vienna, Austria, 19-22 June 2013.

COMMENTS

Background

Single-incision laparoscopic cholecystectomy (SILC) is a novel technique, with safety and efficacy profiles that are comparable to traditional multi-incision laparoscopic cholecystectomy (LC) for uncomplicated benign gallbladder diseases. For complicated gallbladder diseases, such as acute cholecystitis, the published studies regarding SILC have thus far been conducted with only a small number of patients. Studies comparing SILC and traditional LC for acute cholecystitis are rare, but necessary.

Research frontiers

Single-incision laparoscopic surgery (SILS; also known as laparoendoscopic single site surgery) is a novel minimally invasive technique, compared with the traditional multi-incision laparoscopic surgery. Besides the obvious cosmetic advantage (producing no visible scar), decreased post-operative pain and faster recovery are the potential benefits of SILS. However, the higher complication rate that accompanies a beginner operator's learning curve must be accounted for when choosing to apply this technique.

Innovations and breakthroughs

SILC with conventional instruments is as safe and efficacious as traditional multi-incision LC for both complicated and uncomplicated acute cholecystitis when performed by a physician with experienced hands. In particular, the patient benefits are low complication rate and faster postoperative recovery. Before applying SILC in difficult gallbladders, a surgeon must be proficient in this novel technique for simple gallbladder disease and the modified laparoscopic techniques for severe cholecystitis. A low threshold for converting the procedure should be maintained to help ensure patient safety.

Applications

This study suggests that SILC with conventional instruments can be applied to patients with acute cholecystitis safely and efficaciously, particularly when performed by physicians with experienced hands. Better cosmetic outcome and faster recovery time are major advantages.

Terminology

Single-incision laparoscopic surgery is a minimally invasive surgical procedure, in which the surgeon operates through a small single entry site - often the navel. As such, this procedure is considered a type of scarless surgery. The SILS procedure is a good alternative approach (compared to the traditional surgical cholecystectomy procedure) for treating acute cholecystitis, an acute inflammation of the gallbladder characterized by unendurable pain in the right upper abdominal quadrant and is closely correlated with gallbladder stones.

Peer review

This comprehensive comparative study of SILC and the traditional multi-incision LC treatment approach for acute cholecystitis represents the largest case series investigation of SILC in acute cholecystitis published to date. As well as better cosmetic outcome, SILC was shown to have a faster recovery time and less complications. The postoperative complication of fever remains to be fully understood and may be primarily related to the body's inflammatory response to the cholecystitis rather than the surgical procedure itself. Unfortunately, the well known difference in cost between the two procedures and the longer operating time required by SILC make it difficult to justify further prospective studies.

REFERENCES

1 **Markar SR**, Karthikesalingam A, Thrumurthy S, Muirhead L, Kinross J, Paraskeva P. Single-incision laparoscopic surgery (SILS) vs. conventional multiport cholecystectomy: systematic review and meta-analysis. *Surg Endosc* 2012; **26**: 1205-1213 [PMID: 22173546 DOI: 10.1007/s00464-011-2051-0]

2 **Puzziello A**, Orlando G, Siani C, Gervasi R, Lerose MA, Lucisano AM, Vescio G, Sacco R. From 3-port to new laparoendoscopic single-site (LESS) cholecystectomy: a critical analysis of available evidence. *Surg Innov* 2012; **19**: 364-369 [PMID: 22333936 DOI: 10.1177/1553350611436282]

3 **Pisanu A**, Reccia I, Porceddu G, Uccheddu A. Meta-analysis of prospective randomized studies comparing single-incision laparoscopic cholecystectomy (SILC) and conventional multiport laparoscopic cholecystectomy (CMLC). *J Gastrointest Surg* 2012; **16**: 1790-1801 [PMID: 22767084 DOI: 10.1007/s11605-012-1956-9]

4 **Lee PC**, Lo C, Lai PS, Chang JJ, Huang SJ, Lin MT, Lee PH. Randomized clinical trial of single-incision laparoscopic cholecystectomy versus minilaparoscopic cholecystectomy. *Br J Surg* 2010; **97**: 1007-1012 [PMID: 20632264 DOI: 10.1002/bjs.7087]

5 **Chow A**, Purkayastha S, Aziz O, Pefanis D, Paraskeva P. Single-incision laparoscopic surgery for cholecystectomy: a retrospective comparison with 4-port laparoscopic cholecystectomy. *Arch Surg* 2010; **145**: 1187-1191 [PMID: 21173293 DOI: 10.1001/archsurg.2010.267]

6 **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]

7 **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]

8 **Joseph M**, Phillips MR, Farrell TM, Rupp CC. Single incision laparoscopic cholecystectomy is associated with a higher bile duct injury rate: a review and a word of caution. *Ann Surg* 2012; **256**: 1-6 [PMID: 22664556 DOI: 10.1097/SLA.0b013e3182583fde]

9 **Hall TC**, Dennison AR, Bilku DK, Metcalfe MS, Garcea G. Single-incision laparoscopic cholecystectomy: a systematic review. *Arch Surg* 2012; **147**: 657-666 [PMID: 22802063 DOI: 10.1001/archsurg.2012.814]

10 **Antoniou SA**, Pointner R, Granderath FA. Single-incision laparoscopic cholecystectomy: a systematic review. *Surg Endosc* 2011; **25**: 367-377 [PMID: 20607556 DOI: 10.1007/s00464-010-1217-5]

11 **Sasaki K**, Watanabe G, Matsuda M, Hashimoto M. Original single-incision laparoscopic cholecystectomy for acute in-

flammation of the gallbladder. *World J Gastroenterol* 2012; **18**: 944-951 [PMID: 22408354 DOI: 10.3748/wjg.v18.i9.944]
12 **Barbaros U**, Sümer A, Tunca F, Gözkün O, Demirel T, Bilge O, Randazzo V, Dinççağ A, Seven R, Mercan S, Budak D. Our early experiences with single-incision laparoscopic surgery: the first 32 patients. *Surg Laparosc Endosc Percutan Tech* 2010; **20**: 306-311 [PMID: 20975500 DOI: 10.1097/SLE.0b013e3181f20cf6]
13 **Vemulapalli P**, Agaba EA, Camacho D. Single incision laparoscopic cholecystectomy: a single center experience. *Int J Surg* 2011; **9**: 410-413 [PMID: 21515426 DOI: 10.1016/j.ijsu.2011.04.001]
14 **Zubaidi AM**. Single-port laparascopic cholecystectomy: scarless cholecystectomy. *Minim Invasive Surg* 2012; **2012**: 204380 [PMID: 22645675 DOI: 10.1155/2012/204380]
15 **Ikegami T**, Shirabe K, Yoshizumi T, Kayashima H, Maehara Y. Use of the SAND balloon catheter in single-incision laparoscopic cholecystectomy for acute cholecystitis. *Asian J Endosc Surg* 2013; **6**: 134-136 [PMID: 23601999 DOI: 10.1111/ases.12005]
16 **Chuang SH**. From multi-incision to single-incision laparoscopic cholecystectomy step-by-step: one surgeon's self-taught experience and retrospective analysis. *Asian J Surg* 2013; **36**: 1-6 [PMID: 23270818 DOI: 10.1016/j.asjsur.2012.06.002]
17 **Overby DW**, Apelgren KN, Richardson W, Fanelli R. SAGES guidelines for the clinical application of laparoscopic biliary tract surgery. *Surg Endosc* 2010; **24**: 2368-2386 [PMID: 20706739 DOI: 10.1007/s00464-010-1268-7]
18 **Sauerland S**, Agresta F, Bergamaschi R, Borzellino G, Budzynski A, Champault G, Fingerhut A, Isla A, Johansson M, Lundorff P, Navez B, Saad S, Neugebauer EA. Laparoscopy for abdominal emergencies: evidence-based guidelines of the European Association for Endoscopic Surgery. *Surg Endosc* 2006; **20**: 14-29 [PMID: 16247571]
19 **Yamashita Y**, Takada T, Kawarada Y, Nimura Y, Hirota M, Miura F, Mayumi T, Yoshida M, Strasberg S, Pitt HA, de Santibanes E, Belghiti J, Büchler MW, Gouma DJ, Fan ST, Hilvano SC, Lau JW, Kim SW, Belli G, Windsor JA, Liau KH, Sachakul V. Surgical treatment of patients with acute cholecystitis: Tokyo Guidelines. *J Hepatobiliary Pancreat Surg* 2007; **14**: 91-97 [PMID: 17252302]
20 **Clavien PA**, Sanabria JR, Strasberg SM. Proposed classification of complications of surgery with examples of utility in cholecystectomy. *Surgery* 1992; **111**: 518-526 [PMID: 1598671]
21 **Dindo D**, Demartines N, Clavien PA. Classification of surgical complications: a new proposal with evaluation in a cohort of 6336 patients and results of a survey. *Ann Surg* 2004; **240**: 205-213 [PMID: 15273542]
22 **Strasberg SM**, Hertl M, Soper NJ. An analysis of the problem of biliary injury during laparoscopic cholecystectomy. *J Am Coll Surg* 1995; **180**: 101-125 [PMID: 8000648]
23 **Sinha I**, Smith ML, Safranek P, Dehn T, Booth M. Laparoscopic subtotal cholecystectomy without cystic duct ligation. *Br J Surg* 2007; **94**: 1527-1529 [PMID: 17701938]
24 **Beldi G**, Glättli A. Laparoscopic subtotal cholecystectomy for severe cholecystitis. *Surg Endosc* 2003; **17**: 1437-1439 [PMID: 12799885]
25 **Cox MR**, Wilson TG, Luck AJ, Jeans PL, Padbury RT, Toouli J. Laparoscopic cholecystectomy for acute inflammation of the gallbladder. *Ann Surg* 1993; **218**: 630-634 [PMID: 8239777]
26 **Navez B**, Mutter D, Russier Y, Vix M, Jamali F, Lipski D, Cambier E, Guiot P, Leroy J, Marescaux J. Safety of laparoscopic approach for acute cholecystitis: retrospective study of 609 cases. *World J Surg* 2001; **25**: 1352-1356 [PMID: 11596902]
27 **Eldar S**, Sabo E, Nash E, Abrahamson J, Matter I. Laparoscopic cholecystectomy for acute cholecystitis: prospective trial. *World J Surg* 1997; **21**: 540-545 [PMID: 9204745]
28 **Platell C**, Hall JC. Atelectasis after abdominal surgery. *J Am Coll Surg* 1997; **185**: 584-592 [PMID: 9404885]
29 **Hasukić S**, Mesić D, Dizdarević E, Keser D, Hadziselimović S, Bazardzanović M. Pulmonary function after laparoscopic and open cholecystectomy. *Surg Endosc* 2002; **16**: 163-165 [PMID: 11961630]
30 **Karayiannakis AJ**, Makri GG, Mantzioka A, Karousos D, Karatzas G. Postoperative pulmonary function after laparoscopic and open cholecystectomy. *Br J Anaesth* 1996; **77**: 448-452 [PMID: 8942326]
31 **Frazee RC**, Roberts JW, Okeson GC, Symmonds RE, Snyder SK, Hendricks JC, Smith RW. Open versus laparoscopic cholecystectomy. A comparison of postoperative pulmonary function. *Ann Surg* 1991; **213**: 651-653; discussion 653-654 [PMID: 1828139]
32 **Dauleh MI**, Rahman S, Townell NH. Open versus laparoscopic cholecystectomy: a comparison of postoperative temperature. *J R Coll Surg Edinb* 1995; **40**: 116-118 [PMID: 7776273]
33 **Hernandez J**, Ross S, Morton C, McFarlin K, Dahal S, Golkar F, Albrink M, Rosemurgy A. The learning curve of laparoendoscopic single-site (LESS) cholecystectomy: definable, short, and safe. *J Am Coll Surg* 2010; **211**: 652-657 [PMID: 20851645 DOI: 10.1016/j.jamcollsurg.2010.07.008]
34 **Qiu Z**, Sun J, Pu Y, Jiang T, Cao J, Wu W. Learning curve of transumbilical single incision laparoscopic cholecystectomy (SILS): a preliminary study of 80 selected patients with benign gallbladder diseases. *World J Surg* 2011; **35**: 2092-2101 [PMID: 21660626 DOI: 10.1007/s00268-011-1144-1]
35 **Borzellino G**, Sauerland S, Minicozzi AM, Verlato G, Di Pietrantonj C, de Manzoni G, Cordiano C. Laparoscopic cholecystectomy for severe acute cholecystitis. A meta-analysis of results. *Surg Endosc* 2008; **22**: 8-15 [PMID: 17704863]
36 **Rattner DW**, Ferguson C, Warshaw AL. Factors associated with successful laparoscopic cholecystectomy for acute cholecystitis. *Ann Surg* 1993; **217**: 233-236 [PMID: 8452401]

P- Reviewers: Meshikhes AWN, Nash GF **S- Editor:** Zhai HH
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.v19.i43.7751

World J Gastroenterol 2013 November 21; 19(43): 7751-7757
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Transanal natural orifice specimen extraction for laparoscopic anterior resection in rectal cancer

Fang-Hai Han, Li-Xin Hua, Zhi Zhao, Jian-Hai Wu, Wen-Hua Zhan

Fang-Hai Han, Li-Xin Hua, Zhi Zhao, Jian-Hai Wu, Wen-Hua Zhan, Department of Gastrointestinal Surgery, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510080, Guangdong Province, China
Author contributions: Han FH and Zhan WH designed research; Han FH, Hua LX and Zhan WH performed research; Zhao Z provided new reagents or analytic tools; and Wu JH analyzed data; Han FH and Hua LX wrote the paper.
Correspondence to: Wen-Hua Zhan, Professor, Department of Gastrointestinal Surgery, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510080, Guangdong Province, China. fh_han@163.com
Telephone: +86-20-28823388 Fax: +86-20-28823388
Received: April 17, 2013 Revised: May 13, 2013
Accepted: July 17, 2013
Published online: November 21, 2013

Abstract

AIM: To investigate whether transanal natural orifice specimen extraction (NOSE) is a better technique for rectal cancer resection.

METHODS: A prospectively designed database of a consecutive series of patients undergoing laparoscopic low anterior resection for rectal cancer with various tumor-node-metastasis classifications from March 2011 to February 2012 at the First Affiliated Hospital of Sun Yat-Sen University was analyzed. Patient selection for transanal specimen extraction and intracorporeal anastomosis was made on the basis of tumor size and distance of rectal lesions from the anal verge. Demographic data, operative parameters, and postoperative outcomes were assessed.

RESULTS: None of the patients was converted to laparotomy. Respectively, there were 16 cases in the low anastomosis and five in the ultralow anastomosis groups. Mean age of the patients was 45.4 years, and mean body mass index was 23.1 kg/m^2. Mean distance of the lower edge of the lesion from the anal verge was 8.3 cm. Mean operating time was 132 min, and mean intraoperative blood loss was 84 mL. According to the principle of rectal cancer surgery, we performed D2 lymph node dissection in 13 cases and D3 in eight. Mean lymph nodes harvest was 17.8, and the number of positive lymph nodes was 3.4. Median hospital stay was 6.7 d. No serious postoperative complication occurred except for one anastomotic leakage. All patients remained disease free. Mean Wexner score was 3.7 at 11 mo after the operation.

CONCLUSION: Transanal NOSE for total laparoscopic low/ultralow anterior resection is feasible, safe and oncologically sound. Further studies with long-term outcomes are needed to explore its potential advantages.

Key words: Transanal specimen extraction; Natural orifice specimen extraction; Laparoscopic anterior resection; Low/ultra-low anastomosis; Total mesorectal excision

Core tip: Natural orifice specimen extraction (NOSE) is an emerging technique that has been recently applied to the field of rectal cancer resection. However, which is the better approach for rectal cancer remains controversial. In this paper, we present our surgical technique and short-term outcomes of transanal NOSE in total laparoscopic low/ultralow anterior resection (L-AR) for patients with rectal cancer. Based on our limited experience, transanal NOSE in L-AR for rectal cancer is feasible, safe and oncologically sound.

Han FH, Hua LX, Zhao Z, Wu JH, Zhan WH. Transanal natural orifice specimen extraction for laparoscopic anterior resection in rectal cancer. *World J Gastroenterol* 2013; 19(43): 7751-7757 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7751.htm DOI: http://dx.doi.org/10.3748/wjg.v19.

i43.7751

INTRODUCTION

The incidence of rectal cancer is higher in Asia compared with western countries[1]. Technically, resection of low rectal cancer may be the most difficult among all colorectal operations.

At present, traditional colorectal surgery has increasingly given way to laparoscopic anterior resection with total mesorectal excision (L-AR/TME). Evidence-based medicine has established that L-AR/TME is a feasible surgical approach for managing rectal cancer. There were similar results in recent short-term therapeutic effects, local recurrence rate and postoperative survival rate between laparoscopic surgery (LS) and traditional open surgery for radical colon cancer[2]. Meanwhile, the mean estimated blood loss, discharge time after operation, and postoperative hospital stay were significantly reduced in the LS[3]. However, incision of the abdomen is still necessary in order to remove the specimens in LS, which could cause incision infection and increase the incidence of incisional hernia[4].

Natural orifice specimen extraction (NOSE) may be an effective way to address the challenge. NOSE is feasible and safe technically for radical colorectal cancer surgery by traditional laparoscopic techniques, and for removal of the specimens through a natural orifice[5,6]

Total laparoscopic hemicolectomy has been performed successfully by transvaginal NOSE[7]. However, due to its innate limitations, transvaginal NOSE is difficult for radical rectal cancer surgery, especially in low rectal cancer. Here, we introduce a technique used for the laparoscopic radical rectal surgery with TME, and the specimen was removed and anastomosis was accomplished through the anus.

MATERIALS AND METHODS

This study was approved by the ethics committee of the hospital and written informed consent was obtained from the patients. Twenty-one patients with rectal adenocarcinoma underwent the procedure from March 2011 to February 2012 (Table 1). All patients with preoperative diagnosis of rectal cancer were confirmed by endoscopic colonoscopy, pathology, endosonography, and staged by specialized oncologists at our hospital, and preoperatively managed following the guidelines of the National Comprehensive Cancer Network (NCCN). All operations were performed by a single surgeon who was proficient in various laparoscopic colorectal procedures and laparotomy at our hospital.

These patients with tumor stage T4, tumor covering over half of the circumference of the rectum, metastasis in the liver or lungs on preoperative imaging assessment, or body mass index > 28 were excluded.

Three patients whose tumor-node-metastasis classification of T3 was confirmed by endosonography, magnetic resonance imaging (MRI) and computed tomography (CT) received three cycles of chemotherapy prior to surgery. Radiotherapy followed by resection was conducted based on the national guidelines. The feasibility of the surgery was reappraised at 2 wk after the treatment. All three patients had symptom relief, the tumor was reduced in size, and there were limited side effects of neoadjuvant chemotherapy.

Table 1 Patient demographic data

Patient demographic data	Value
Age (yr)	45.4 ± 3.6
Body mass index	23.1 ± 2.8
Sex (male/female ratio)	12/9
Mean wexner score	3.7 ± 1.6

Wexner Score was obtained in follow-up at 6 mo.

The day before the operation, all patients underwent systemic bowel preparation, and used prophylactic antibiotics.

Surgical procedure

Laparoscopic phase. The patient was positioned in a modified lithotomy position, and the abdomen was then insufflated with 10-12 mmHg CO_2. Four ports were used in the following procedure. The first port was a 12-mm blunt-tip for the laparoscope, which was placed in the umbilicus using the minilaparotomy technique. The second to fourth ports were 10-mm operating ports in the right lower quadrant, and two 5-mm ports in the right middle abdomen and left lower quadrant, respectively.

Colon mobilization, lymph node dissection, and mesenteric excision were performed laparoscopically in the usual manner. First, the sacral promontory was separated by ultrasonic scalpel (Harmonic ACE; Ethicon Endo-Surgery, OH, United States) from the right side of the rectum. Second, before the tumor was mobilized, the inferior mesenteric artery was ligated at its point of pedicle from the aorta with a large or oversized Hem-o-lock. The I -Ⅲ branches of artery and vein of sigmoid were cut off while the marginal artery of the proximal colon was preserved. Next, the inferior mesenteric vein was ligated at the corresponding height to the artery. We mobilized the splenic flexure in two patients because there were existing tensions in the anastomosis. Third, the posterior mesorectal fascia was identified and the dissection was extended to the level of the sacral promontory in the avascular plane. The rectum was fully dissociated to the levator ani muscle plane as far as possible along the Denonvillier's fascia. The fragment of the distal rectum that was located 2 cm above the tumor was clamped with a detachable clip.

We preserved the inferior hypogastric nerves as far as possible during the procedure.

Perineal phase. The anus was dilated gently until it

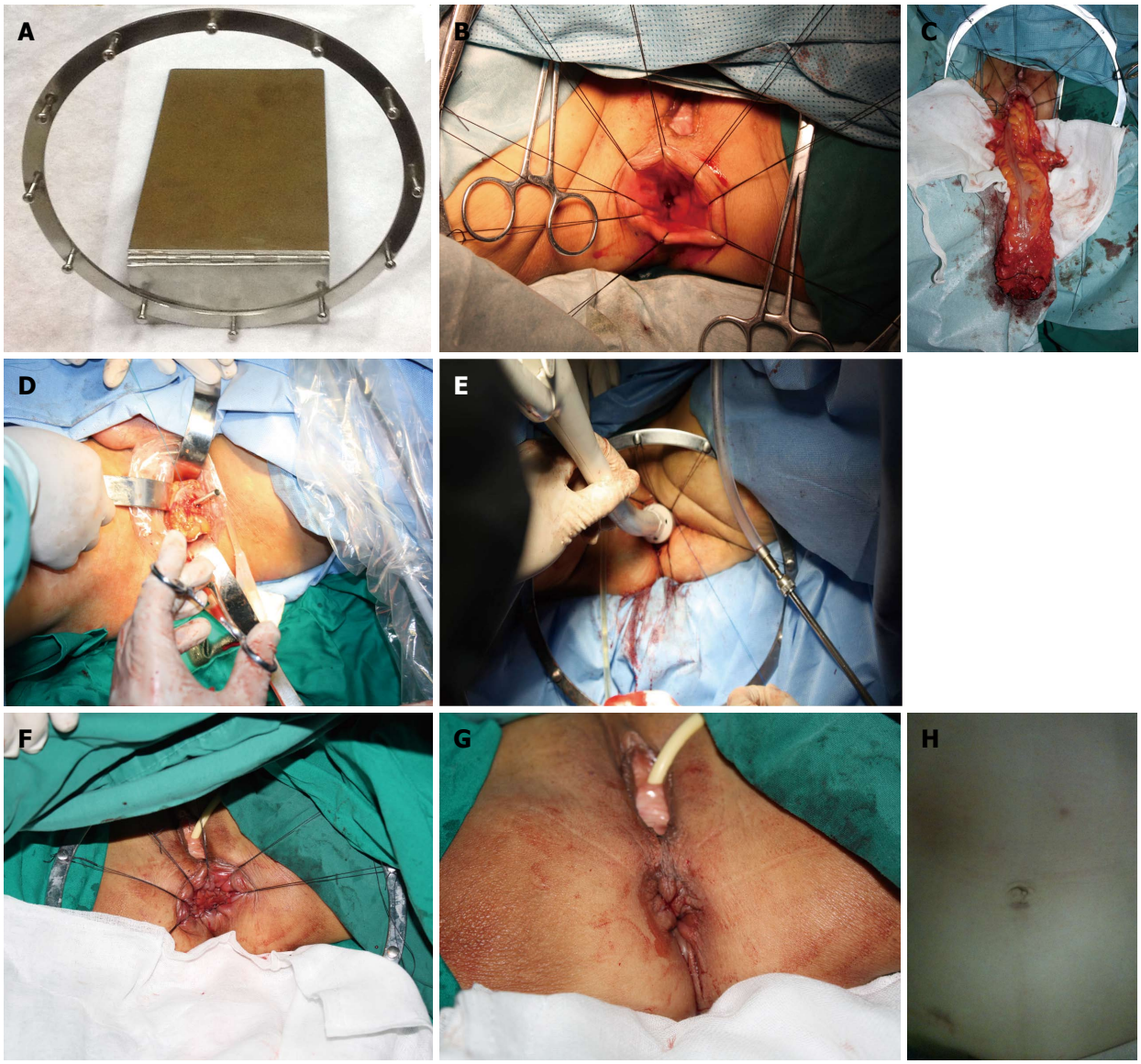


Figure 1 Surgical procedure. A, B: Dilating the anus with a home-made dilator in which the bottom can be folded; C: Exteriorizing the specimen through the anus; D: Placing the anvil shaft into the stump of the proximal colon; E: Placing the anastomosis body into the anus; F: Completion of manual anastomosis procedure; G: Postoperative appearance of the anus; H: Appearance of abdominal wall 3 mo after surgery.

could accommodate four fingers. A home-made anus dilator and fine silk traction sutures were placed into the proximal lip of the exposed mucosal edge at a vertical orientation, in order to expand the anus and expose the rectum (Figure 1A and B). The level of intended transection had to maintain a margin of 2 cm distal from the tumor[8]. After irrigating the rectum with 1 L diluted povidone-iodine solution, we sutured two parallel circle-purse-strings in the distal rectal wall with 2-0 prolene lines through the dilated anus. The upper one maintained a minimal margin of 1-1.5 cm distal from the tumor, while the lower was located in the rectal mucosa at 1 cm above the dentate line. Between the two circle-purse-strings, full-thickness rectal circumferential dissection was extended by ultrasonic scalpel. At this point, the peritoneal cavity was extended circumferentially cephalad as far as possible, and then joined the perineal and laparoscopic dissection planes.

The stump of the proximal rectosigmoid was exteriorized through the dilated anus and opened stump of the distal rectum (Figure 1C). After clamping with purse string forceps, the section of proximal colon, which had to be maintained at a minimal margin of 10 cm above the tumor, was transected under direct vision. After purse-string suturing, an anvil shaft was placed into the stump of the proximal colon, then it was pushed gently back into the peritoneal cavity (Figure 1D). The purse-string suture was tied to the anvil shaft before connecting it to the central shaft of the circular stapler (CDH 29; Ethicon Endo-Surgery, OH, United States). After tightening the lower circle-purse string, the anastomosis was placed into the anus (Figure 1E). Then the anastomat was fired to create an end-to-end coloanal anastomosis in the usual manner[9]. An air test was conducted through the anus.

The stitching was reinforced by bioabsorbable suture if necessary. A pelvic drain was inserted.

We performed the procedure successfully in 16 patients. Due to the low position of the stump of the distal rectum, we performed manual anastomosis and protective loop ileostomy in 5 patients (Figure 1F).

The negative margins were confirmed in all patients by intraoperative frozen biopsy. The mesorectal integrity[10] and circumferential situation[11] of the resected specimens were evaluated by a senior surgeon and qualified pathologist macroscopically and microscopically, in order to ensure that the tumor had been resected completely. The status of the mesorectal specimens was graded into three categories. We differentiated them as complete (intact mesorectum > 5 cm, while defect of mesentery < 5 mm); nearly complete (intact mesorectum > 5 cm, while defect of mesentery > 5 mm); and incomplete (incomplete mesorectum). We defined a positive margin if the circumferential margin from the tumor was < 2 mm under microscopy.

RESULTS

We successfully performed the procedure in all 21 patients, and none of them was converted to laparotomy (Table 2). There were 16 and five patients in the low or ultralow anastomosis groups, respectively. According to macroscopic specimen assessment of TME, the status was complete for 18 patients, while nearly complete for three patients. In addition, the circumferential resection margin was negative in all patients (Table 3).

The mean maximum tumor diameter was 4.6 ± 1.7 cm. According to the principle of rectal cancer surgery and no-touch isolation technique, we performed D2 lymph node dissection in 13 patients and D3 dissection in eight patients. The postoperative course was unremarkable in most patients, with prompt return of bowel activity and short postoperative stay, except for one patient who was complicated by anastomotic leakage (Table 4). Anastomotic leakage was confirmed by stools leaking from a drain. He was treated with nil by mouth, decompression of the rectum by transanal drainage, and antibiotic infusion until the leak healed spontaneously. He was discharged on the postoperative day 15.

According to the guidelines of NCCN, all patients with T3/T4 or postoperative node-positive tumors underwent postoperative chemotherapy for 6-9 cycles. The follow-up period ranged from 11 to 23 mo. Follow-up examinations were scheduled at 2 wk and 1, 2, 3, 6, 9 and 12 mo, and every 6 mo thereafter until 5 years. All patients underwent CT of the chest, abdomen, and pelvis every 6 mo and colonoscopy at 12 mo, but remained disease free. All five patients who had handsewn coloanal anastomosis with a diverting ileostomy had their ileostomies reversed at 3-6 mo after the operation, based on the diagnosis of free from tumor recurrence and anastomotic stenosis, which were confirmed by endoscopic colonoscopy, barium enema, MRI, and CT. Anal continence was measured with the validated Wexner fecal incontinence scoring system (0 = perfect continence, 20 = complete incontinence). The mean Wexner score was 3.7 (range 0-5) at > 11 mo after the operation.

Table 2 Intraoperative information

Intraoperative information	Value
Mean operation time (min)	132 ± 85
Mean intraoperative blood loss(mL)	84 ± 15
Mean tumor diameter (cm)	4.6 ± 1.7
Distance of lesion from anal verge (cm)	8.3 ± 3.5
Protective ileostomy	5 (23.8)
Defecation time after operation (d)	2.5 ± 1.4

Table 3 Patient pathological parameters

Items	Number of cases
Pathological diagnoses	
Well differentiated	10
Poorly differentiated	7
Myxoadenocarcinoma	4
Specimen macro-assessment of TME	21 (radical resection)
Circumferential resection margin	21 (Negative)
Postoperative pathology staging (TNM)	
T1-4N0M0	7
T1-2N1M0	5
T3-4N1M0	6
T3-4N2M0	3
Lymph nodes harvest (mean)	17.8 ± 4.6
Metastatic lymph nodes (mean)	3.4 ± 1.8

TME: Total mesorectal excision; TNM: Tumor node metastasis

DISCUSSION

In the past 10 years, L-AR has been performed at our hospital according to the principle of TME for patients with low rectal cancer. Traditional large abdominal incision has been replaced gradually by small abdominal incision. L-AR benefits patients not only in terms of cosmetics and postoperative rehabilitation, but also in reducing surgical interference, maintaining immune function and homeostasis, rapid recovery, and relieving psychological stress after surgery. However, L-AR is still considered imperfect due to the requirement for abdominal incision of 5-7 cm at minimum to remove the specimen completely. There are still some complications, such as abdominal incision infection, postoperative somatic pain, and incisional hernia[12]. According to bulk analysis of cases, wound infections occurred in 13.5% of patients after L-AR (2.7% trocar and 10.8% extraction site), and incisional hernias developed in 24.3%, and extraction sites accounted for 85.7% of all wound complications[13].

In order to reduce the impact of L-AR incision and eliminate abdominal incision completely, natural orifice transluminal endoscopic surgery (NOTES) has increased in recent years, which can avoid incisional infection and hernia, and achieve better cosmetic results[14,15].

Recently, transvaginal (posterior fornix incision) has been the main approach of NOTES in most colectomy

Table 4 Postoperative complications *n* (%)

Length of hospitalization (d)	6.1 ± 2.7
Postoperative complications	
UTI	2 (9.5)
Anastomotic leakage	1 (4.7)
Anastomotic bleeding	0
Incision infection	0
Intestinal obstruction	0
Impotence	1 (4.7)
Fecal incontinence	0
Anal stenosis	0
Total	4 (18.9)

The data of fecal incontinence, impotence, and anal stenosis was obtained at 1-year follow-up. UTI: Urinary tract infection.

procedures[16-18]. However, there are still some negative factors in low/ultralow rectal cancer which hinder the application of transvaginal approach NOTES. First, there are technical shortcomings, such as lack of experience and technical complexity, additional adjacent organ injury, extended operation time, and specialized equipment requirement, which account for the increased cost of the operation. Second, it is sometimes difficult to remove a larger tumor specimen through the posterior vaginal fornix incision. Third, there are many technical difficulties in achieving sphincter preservation for low/ultralow rectal cancer by the transvaginal approach. Finally, the transvaginal approach is obviously limited to female patients, which is a major hindrance for widespread use of the technique in clinical practice.

As a result, more surgeons have been trying to find new approaches for NOTES in low rectal cancer. With regard to the applicability of NOTES in colorectal surgery, the transanal access route of NOTES is intuitively the optimal one. First, rather than creating an opening through an otherwise healthy organ to perform the rectal anterior resection, enterotomy is carried out on the diseased organ itself. Second, the enterotomy is ultimately closed by incorporating it into a standard colorectal anastomosis, which is the requirement of surgery regardless of whether it is achieved *via* NOTES or standard surgery. Finally, transanal NOTES could have substantial benefits over standard transabdominal approaches[19].

At present, transanal access NOTES in radical colorectal cancer surgery has been completed successfully in animal models, but few surgeons have put it into clinical practice due to potential technical difficulties, such as intra-abdominal intestinal fecal contamination, or increased possibility of infection through the colon lumen. All of these factors may affect the safety of the procedure. For example, clinical reports have confirmed that common complications included wound infection (56.7%), septicemia (31.7%), and enterocutaneous fistula (16.7%) in patients who sustained penetrating colon injuries[20]. However, with the improvement in anatomical techniques and equipment, transanal NOTES has been performed for resection of the rectum in pig models *in vivo* or fresh cadavers[21], as well as laparoscopy-assisted transanal NOTES for left-sided colorectal resection[22] and sigmoidectomy[23]. Unfortunately, these techniques require expensive equipment, which limits the clinical application of NOTES, especially in developing countries.

As a development of NOTES, transanal NOSE is an emerging technique that has been recently applied to the field of rectal excision. Darzi *et al*[24] have described a technique of total laparoscopic left-sided colonic resection and transanal specimen delivery. Franklin *et al*[25] have reported that laparoscopic colectomy in patients with stage Ⅲ colorectal cancer is oncologically adequate. Fukunaga *et al*[26] have performed radical rectal cancer surgery with removal of the specimens through the anus, thus avoiding abdominal incision. Transanal specimen extraction can also resolve the problems found in obese patients with short or hypertrophic mesentery, or deep abdominal wall, which have been challenges for transabdominal specimen removal[27]. It has been confirmed that transanal NOSE is technically feasible. It may be a bridge between NOTES and the conventional laparoscopic approach for radical colorectal cancer surgery.

Our current experience showed that transanal NOSE, combined with TME and L-AR techniques for rectal cancer, could be adapted for radical tumor resection and minimally invasive surgery. Its technical feasibility and oncological principles have been demonstrated by many surgeons[28]. The rectal stump is a "necessary" trauma. We can accurately determine the distal cutting edge of the rectum through the full use the rectal stump. Combination of traditional laparoscopic techniques and removal of specimens through a natural orifice can minimize surgical injury[29]. Traditional laparoscopic surgical techniques provide a large operating space, mature technology and broad vision, which allows one to dissect accurately the mesorectal, pelvic visceral and parietal fascia. We can ensure that the inferior mesenteric artery is ligated at the root, in order to block the tumor blood supply and venous drainage, and minimize the chance of metastasis. Care is required to avoid any injury to the mesenteric arcades so as to guarantee an adequate blood supply to the descending colon. The operation was carried out following the "holy plane", which is placed between the pelvic visceral fascia and rectal fascia propria, and then to the anterior Denonvillier's fascia. The mesorectum should be completely mobilized while the pelvic autonomic nerve is preserved.

After the anus was fully dilated, we used a home-made anal dilator and fine silk traction sutures to evert the anus and expose the rectum, then placed a protective bag into the anus. In the premise of protecting blood supply of the residual colon, the pre-cut specimen was fully freed in the peritoneal cavity, then gently pulled out through the anus.

We paid attention to protecting the functions of anal sphincter while performing a standard radical resection of rectal cancer. Even if the specimen is a relatively large one, for example, the hypertrophic mesorectum, it can be removed smoothly from the fully dilated anus routinely

without tearing the rectum or damaging the anal sphincter. The anus and rectum can be returned to their normal diameter after the operation (Figure 1H).

When the stump of the proximal rectum was exteriorized through the dilated anus and opened stump of the distal rectum, we transected the proximal colorectum under direct vision[30]. After intracorporeal purse-string sutures with 2/0 prolene, we used an anastomat to create an end-to-end coloanal anastomosis in the usual manner. Although some studies have found that the J-pouch is superior to end-to-end reconstruction for low rectal cancer[9,31], the latter resulted in acceptable anal function at 6 mo follow-up in our study, due to the careful protection of the anal sphincter, with no tension and a good blood supply in the anastomotic stoma.

In order to prevent peritoneal seeding and trocar-site metastasis, we implemented the general rules for laparoscopic surgery, such as the no-touch technique, appropriate resection margins, early bagging of the resected specimen, and wound protection into our laparoscopic colorectal procedures. Compared to the traditional laparoscopic techniques, our technique had good cosmetic results and reduced the chance of metastasis in the abdominal wall, without increasing complications[32].

Laurent *et al*[33] reported that the conversion rate of laparoscopic radical resection for low rectal cancer was 15.5%. The conversion rate was higher due to the difficulties experienced in fixing colorectal, separating in the pelvic, unexpected intraoperative bleeding, and failure of the closure device, or anastomosis. However, such difficulties did not jeopardize our treatment due to the elasticity and compliance of the tissue while we used mature laparoscopic techniques to remove the specimens through the anus. With full use of the natural orifice of the anus and rectum, total laparoscopic rectal resection is feasible and safe. Such a technique decreases the abdominal surgery complications, and maintains the operation time and the cost of surgery to those of standard L-AR. It also provides significant improvement of the traditional laparoscopic techniques.

However, the present surgical indications are limited to patients with early cancer. Mesorectal invasion and tumor diameter > 6 cm are not included here due to the lack of a large randomized controlled study for this procedure. The operation field is narrowed and the vision is not clear through the anal approach in some conditions, such as a narrow pelvis or large tumor. Although there are reports of microsurgical resection through the anus[34], there is no specialized surgical instrument to complete the procedures for anus dilation, specimen removal, and distal suturing. There is urgency to develop better-adapted tools such as a modified flexible transanal endoscopic platform, longer and more flexible dissecting instruments, staplers and hemostatic devices to permit safe completion of these procedures without any transabdominal assistance. This technique requires further regulation and improvement.

In our limited experience, transanal specimen extraction in total laparoscopic low/ultralow anterior resection is feasible, safe, and oncologically sound for selected cases. The majority of patients have an acceptable functional outcome. Further studies with long-term outcomes are needed to explore the potential advantages of this technique.

COMMENTS

Background

The incidence of rectal cancer is higher in Asia compared with western countries. Technically, the resection of low rectal cancer may be one of the most difficult among all colorectal surgery procedures.

Research frontiers

At present, traditional colorectal surgery has increasingly given way to laparoscopic anterior resection with total mesorectal excision (L-AR/TME). Evidence-based medicine has established that L-AR/TME is a feasible surgical approach for managing rectal cancer. There have been similar results recently for short-term therapeutic effects, local recurrence rate, and postoperative survival rate between laparoscopic surgery and traditional open surgery for radical colon cancer.

Innovations and breakthroughs

This study showed that transanal specimen extraction by total laparoscopic low/ultralow anterior resection is feasible, safe, and oncologically sound for selected cases. The majority of patients had an acceptable functional outcome.

Applications

There is an urgency to develop better adapted tools such as a modified flexible transanal endoscopic platform, longer and more flexible dissecting instruments, and staplers and hemostatic devices to permit safe completion of these procedures without any transabdominal assistance. This technique needs further standardization and improvement.

Peer review

This study was interesting and highly innovative in terms of colorectal surgical technique, especially for a surgical rather than gastroenterological audience.

REFERENCES

1 **Kim DH**, Shin MH, Ahn YO. Incidence pattern of colorectal cancer in Korea by subsite of origin. *J Korean Med Sci* 2000; **15**: 675-681 [PMID: 11194194]

2 **Lee JE**, Joh YG, Yoo SH, Jeong GY, Kim SH, Chung CS, Lee DG, Kim SH. Long-term Outcomes of Laparoscopic Surgery for Colorectal Cancer. *J Korean Soc Coloproctol* 2011; **27**: 64-70 [PMID: 21602964 DOI: 10.3393/jksc.2011.27.2.64]

3 **Liberman MA**, Phillips EH, Carroll BJ, Fallas M, Rosenthal R. Laparoscopic colectomy vs traditional colectomy for diverticulitis. Outcome and costs. *Surg Endosc* 1996; **10**: 15-18 [PMID: 8711597]

4 **Vestweber B**, Galetin T, Lammerting K, Paul C, Giehl J, Straub E, Kaldowski B, Alfes A, Vestweber KH. Single-incision laparoscopic surgery: outcomes from 224 colonic resections performed at a single center using SILS. *Surg Endosc* 2013; **27**:434-442 [PMID: 22806519 DOI: 10.1007/s00464-012-2454-6]

5 **Whiteford MH**, Spaun GO. A colorectal surgeons viewpoint on natural orifice translumenal endoscopic surgery. *Minerva Chir* 2008; **63**: 385-388 [PMID: 18923349]

6 **Takayama S**, Hara M, Sato M, Takeyama H. Hybrid natural orifice transluminal endoscopic surgery for ileocecal resection. *World J Gastrointest Surg* 2012; **4**: 41-44 [PMID: 22408718 DOI: 10.4240/wjgs.v4.i2.41]

7 **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]

8 **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]

9 **Wexner SD**. Restorative proctectomy with colon pouch-anal anastomosis by laparoscopic transanal pull-through: an available option for low rectal cancer? *Surg Endosc* 2007; **21**: 1679 [PMID: 17593441]

10 **García-Granero E**, Faiz O, Muñoz E, Flor B, Navarro S, Faus C, García-Botello SA, Lledó S, Cervantes A. Macroscopic assessment of mesorectal excision in rectal cancer: a useful tool for improving quality control in a multidisciplinary team. *Cancer* 2009; **115**: 3400-3411 [PMID: 19479978 DOI: 10.1002/cncr.24387]

11 **Wiggers T**, van de Velde CJ. The circumferential margin in rectal cancer. Recommendations based on the Dutch Total Mesorectal Excision Study. *Eur J Cancer* 2002; **38**: 973-976 [PMID: 11978522]

12 **Ihedioha U**, Mackay G, Leung E, Molloy RG, O'Dwyer PJ. Laparoscopic colorectal resection does not reduce incisional hernia rates when compared with open colorectal resection. *Surg Endosc* 2008; **22**: 689-692 [PMID: 17623241]

13 **Winslow ER**, Fleshman JW, Birnbaum EH, Brunt LM. Wound complications of laparoscopic vs open colectomy. *Surg Endosc* 2002; **16**: 1420-1425 [PMID: 12085142]

14 **Gettman MT**, Lotan Y, Napper CA, Cadeddu JA. Transvaginal laparoscopic nephrectomy: development and feasibility in the porcine model. *Urology* 2002; **59**: 446-450 [PMID: 11880100]

15 **Kalloo AN**, Singh VK, Jagannath SB, Niiyama H, Hill SL, Vaughn CA, Magee CA, Kantsevoy SV. Flexible transgastric peritoneoscopy: a novel approach to diagnostic and therapeutic interventions in the peritoneal cavity. *Gastrointest Endosc* 2004; **60**: 114-117 [PMID: 15229442]

16 **Gettman MT**, Blute ML. Transvesical peritoneoscopy: initial clinical evaluation of the bladder as a portal for natural orifice translumenal endoscopic surgery. *Mayo Clin Proc* 2007; **82**: 843-845 [PMID: 17605965]

17 **Lacy AM**, Delgado S, Rojas OA, Almenara R, Blasi A, Llach J. MA-NOS radical sigmoidectomy: report of a transvaginal resection in the human. *Surg Endosc* 2008; **22**: 1717-1723 [PMID: 18461385 DOI: 10.1007/s00464-008-9956-2]

18 **Wilson JI**, Dogiparthi KK, Hebblethwaite N, Clarke MD. Laparoscopic right hemicolectomy with posterior colpotomy for transvaginal specimen retrieval. *Colorectal Dis* 2007; **9**: 662 [PMID: 17824986]

19 **Dozois EJ**, Larson DW, Dowdy SC, Poola VP, Holubar SD, Cima RR. Transvaginal colonic extraction following combined hysterectomy and laparoscopic total colectomy: a natural orifice approach. *Tech Coloproctol* 2008; **12**: 251-254 [PMID: 18679569 DOI: 10.1007/s10151-008-0428-4]

20 **Adesanya AA**, Ekanem EE. A ten-year study of penetrating injuries of the colon. *Dis Colon Rectum* 2004; **47**: 2169-2177 [PMID: 15657670]

21 **Bhattacharjee HK**, Buess GF, Becerra Garcia FC, Storz P, Sharma M, Susanu S, Kirschniak A, Misra MC. A novel single-port technique for transanal rectosigmoid resection and colorectal anastomosis on an ex vivo experimental model. *Surg Endosc* 2011; **25**: 1844-1857 [PMID: 21136108 DOI: 10.1007/s00464-010-1476-1]

22 **Costantino FA**, Diana M, Wall J, Leroy J, Mutter D, Marescaux J. Prospective evaluation of peritoneal fluid contamination following transabdominal vs. transanal specimen extraction in laparoscopic left-sided colorectal resections. *Surg Endosc* 2012; **26**: 1495-1500 [PMID: 22179455 DOI: 10.1007/s00464-011-2066-6]

23 **Leroy J**, Diana M, Wall J, Costantino F, D'Agostino J, Marescaux J. Laparo-endoscopic single-site (LESS) with transanal natural orifice specimen extraction (NOSE) sigmoidectomy: a new step before pure colorectal natural orifices transluminal endoscopic surgery (NOTES®). *J Gastrointest Surg* 2011; **15**: 1488-1492 [PMID: 21584823]

24 **Darzi A**, Super P, Guillou PJ, Monson JR. Laparoscopic sigmoid colectomy: total laparoscopic approach. *Dis Colon Rectum* 1994; **37**: 268-271 [PMID: 8137674]

25 **Franklin ME**, Kazantsev GB, Abrego D, Diaz-E JA, Balli J, Glass JL. Laparoscopic surgery for stage III colon cancer: long-term follow-up. *Surg Endosc* 2000; **14**: 612-616 [PMID: 10948295]

26 **Fukunaga M**, Kidokoro A, Iba T, Sugiyama K, Fukunaga T, Nagakari K, Suda M, Yoshikawa S. Laparoscopy-assisted low anterior resection with a prolapsing technique for low rectal cancer. *Surg Today* 2005; **35**: 598-602 [PMID: 15976960]

27 **Raftopoulos I**, Courcoulas AP, Blumberg D. Should completely intracorporeal anastomosis be considered in obese patients who undergo laparoscopic colectomy for benign or malignant disease of the colon? *Surgery* 2006; **140**: 675-682; discussion 682-683 [PMID: 17011916]

28 **Ohtani H**, Tamamori Y, Azuma T, Mori Y, Nishiguchi Y, Maeda K, Hirakawa K. A meta-analysis of the short- and long-term results of randomized controlled trials that compared laparoscopy-assisted and conventional open surgery for rectal cancer. *J Gastrointest Surg* 2011; **15**: 1375-1385 [PMID: 21557014 DOI: 10.1007/s11605-011-1547-1]

29 **Sylla P**, Willingham FF, Sohn DK, Gee D, Brugge WR, Rattner DW. NOTES rectosigmoid resection using transanal endoscopic microsurgery (TEM) with transgastric endoscopic assistance: a pilot study in swine. *J Gastrointest Surg* 2008; **12**: 1717-1723 [PMID: 18704596 DOI: 10.1007/s11605-008-0637-1]

30 **Nagtegaal ID**, Quirke P. What is the role for the circumferential margin in the modern treatment of rectal cancer? *J Clin Oncol* 2008; **26**: 303-312 [PMID: 18182672]

31 **Steffen T**, Tarantino I, Hetzer FH, Warschkow R, Lange J, Zünd M. Safety and morbidity after ultra-low coloanal anastomoses: J-pouch vs end-to-end reconstruction. *Int J Colorectal Dis* 2008; **23**: 277-281 [PMID: 18071719]

32 **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]

33 **Laurent C**, Leblanc F, Gineste C, Saric J, Rullier E. Laparoscopic approach in surgical treatment of rectal cancer. *Br J Surg* 2007; **94**: 1555-1561 [PMID: 17668915]

34 **Darwood RJ**, Wheeler JM, Borley NR. Transanal endoscopic microsurgery is a safe and reliable technique even for complex rectal lesions. *Br J Surg* 2008; **95**: 915-918 [PMID: 18496889 DOI: 10.1002/bjs.6018]

P- Reviewers: Kirshtein B, Kim YJ, Leitman M, M'Koma A
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.v19.i43.7758

World J Gastroenterol 2013 November 21; 19(43): 7758-7765
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

MicroRNA-143 suppresses gastric cancer cell growth and induces apoptosis by targeting COX-2

Xiao-Li Wu, Bin Cheng, Pei-Yuan Li, Huan-Jun Huang, Qiu Zhao, Zi-Li Dan, Dean Tian, Peng Zhang

Xiao-Li Wu, Bin Cheng, Pei-Yuan Li, Huan-Jun Huang, Qiu Zhao, Zi-Li Dan, Dean Tian, Department of Gastroenterology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China
Peng Zhang, Department of Gastrointestinal Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China
Author contributions: Wu XL performed the majority of experiments and wrote the manuscript; Cheng B, Li PY, Huang HJ, Zhao Q and Dan ZL provided vital reagents and analytical tools; Tian D provided financial support for this work; and Zhang P designed the study and edited the manuscript.
Correspondence to: Peng Zhang, MD, PhD, Department of Gastrointestinal Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Avenue, Wuhan 430030, Hubei Province, China. zhangpeng@tjh.tjmu.edu.cn
Telephone: +86-27-83665256 Fax: +86-27-83665256
Received: May 30, 2013 Revised: September 20, 2013
Accepted: September 29, 2013
Published online: November 21, 2013

Abstract

AIM: To investigate the function of microRNA-143 (miR-143) in gastric cancer and explore the target genes of miR-143.

METHODS: A quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed to evaluate miR-143 expression in gastric cancer cell lines. After transfecting gastric cancer cells with miR-143-5p and miR-143-3p precursors, Alamar blue and apoptosis assays were used to measure the respective proliferation and apoptosis rates. Cyclooxygenase-2 (COX-2) expression was determined by real-time RT-PCR and Western blot assays after miR-143 transfection. Reporter plasmids were constructed, and a luciferase reporter assay was used to identify the miR-143 binding site on COX-2.

RESULTS: Both miR-143-5p and miR-143-3p were significantly downregulated in multiple gastric cancer cell lines. Forced miR-143-5p and miR-143-3p expression in gastric cancer cells produced a profound cytotoxic effect. MiR-145-5p transfection into gastric cancer cells resulted in a greater growth inhibitory effect (61.23% ± 3.16% *vs* 46.58% ± 4.28%, $P < 0.05$ in the MKN-1 cell line) and a higher apoptosis rate (28.74% ± 1.93% *vs* 22.13% ± 3.31%, $P < 0.05$ in the MKN-1 cell line) than miR-143-3p transfection. Further analysis indicated that COX-2 expression was potently suppressed by miR-143-5p but not by miR-143-3p. The activity of a luciferase reporter construct that contained the 3′-untranslated region (UTR) of COX-2 was downregulated by miR-143-5p (43.6% ± 4.86%, $P < 0.01$) but not by miR-143-3p. A mutation in the miR-145-5p binding site completely ablated the regulatory effect on luciferase activity, which suggests that there is a direct miR-145-5p binding site in the 3′-UTR of COX-2.

CONCLUSION: Both miR-143-5p and miR-143-3p function as anti-oncomirs in gastric cancer. However, miR-143-5p alone directly targets COX-2, and it exhibits a stronger tumor suppressive effect than miR-143-3p.

Key words: Gastric cancer; MicroRNA-143; Anti-oncomir; Cyclooxygenase-2; Apoptosis

Core tip: MicroRNA-143 (miR-143) has been reported to be a tumor suppressor. However, the functions of miR-143-5p and miR-143-3p have never been compared. In this study, we found that both miR-143-5p and miR-143-3p function as tumor suppressors in gastric cancer; however, miR-143-5p alone directly targets cyclooxygenase-2, and it exhibits a stronger tumor suppressive effect than miR-143-3p.

Wu XL, Cheng B, Li PY, Huang HJ, Zhao Q, Dan ZL, Tian D,

Zhang P. MicroRNA-143 suppresses gastric cancer cell growth and induces apoptosis by targeting COX-2. *World J Gastroenterol* 2013; 19(43): 7758-7765 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7758.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7758

INTRODUCTION

MicroRNAs (miRNAs) are short (19-24 nt) non-coding RNAs that control target mRNA translation and stability by binding to regulatory sites that are mostly located in the 3'-untranslated region (UTR) of transcripts[1]. Numerous miRNAs have been shown to display tumor suppressor activity, while others reportedly act as oncogenes[2]. The expression levels of these RNAs are altered in many human tumors, resulting in distinct miRNA networks in various tumor types[3]. Some targets of these miRNAs have been identified, but many of the critical cancer proteins and pathways that they regulate remain unknown.

MiR-143 is considered a pivotal regulator of gene expression because it directly targets multiple mRNAs that code proteins involved in cell proliferation, differentiation, survival and apoptosis, including cyclooxygenase-2 (COX-2)[4], V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS)[5,6], B-cell lymphoma 2 (Bcl-2)[7,8], plasminogen activator inhibitor-1 (PAI-1)[9], myosin VI (MYO6)[10], matrix metallopeptidase 13 (MMP-13)[11], DNA (cytosine-5)-methyltransferase 3A (DNMT3A)[12] and E twenty-six-like transcription factor 1 (ELK1)[13]. The relevance of miR-143 as a putative cancer biomarker is increasing, because this miRNA is downregulated in various human tumors and can suppress tumor growth in cancers of the urogenital system[7,9,10], digestive system[14-16], respiratory system[17,18] and nervous system[19], as well as some sarcomas[11,20] and B-cell malignancies[21]. MiR-143 expression has also been reported to be downregulated in human gastric cancer tissues and cell lines[22]. The expression of miR-143 is significantly decreased in stage Ⅳ gastric cancer, compared to stages Ⅰ and Ⅱ cancers[23]. However, the role of miR-143 in gastric cancer and the underlying mechanisms require further investigation.

Among the target genes regulated by miR-143, COX-2 is particularly important. COX, also known as prostaglandin (PG) H2 synthase, is the rate-limiting enzyme in the conversion of arachidonic acid into PGs. COX-2 expression in cells and animal models is associated with tumor cell growth and metastasis, enhanced cellular adhesion and apoptosis inhibition[24]. Pharmacologic inhibitors of COX-2 can decrease the growth of certain human tumors[25,26] and prevent tumorigenesis in animal models[27]. A pathological study showed increased COX-2 expression levels in gastric cancer[28,29]. Reduced COX-2 expression in gastric cancer cells led to markedly decreased proliferation and metastatic capability, demonstrating that COX-2 activity is necessary for gastric cancer cell proliferation and metastasis[30]. All of the above evidence indicates that COX-2 plays an important role in gastric cancer. In this study, we investigated the roles of miR-143 and COX-2 in gastric cancer and found that both miR-143-5p and miR-143-3p function as tumor suppressors in gastric cancer; however, miR-143-5p alone directly targeted COX-2 and exhibited a stronger tumor suppressive effect than miR-143-3p.

MATERIALS AND METHODS

Cell culture

The human gastric cancer cell lines MKN-1, MKN-7, AGS, SGC-7901 and BGC-823 and the normal gastric epithelium cell line GES-1 were grown in RPMI 1640 medium supplemented with 10% FBS (Hyclone). The cell cultures were incubated in room air at 37 ℃ in a humidified atmosphere of 5% CO_2.

Reverse transcription and real-time polymerase chain reaction to quantify mature miR-143

Total RNA was extracted with TRIzol (Invitrogen). For mature miRNA expression analysis, cDNA was synthesized with the Taqman MiRNA Reverse Transcription kit (Applied Biosystems) and 100 ng of total RNA (100 ng/μL), along with 1 μL of miR-143-5p (Applied Biosystems) or miR-143-3p (Applied Biosystems) specific primers that were supplied with the miRNA Taqman MicroRNA Assay, according to the manufacturer's instructions. Quantitative real-time polymerase chain reaction (PCR) analyses were performed in triplicate on a 7900HT Real-Time PCR System (Applied Biosystems), and the data were normalized to RNU6B (Applied Biosystems) for each reaction. The thermal cycling profile used was as follows: 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 60 s. Quantification was performed according to the standard $^{\Delta\Delta}$CT method.

Transfection of the miR-143 precursor

Cells were seeded 24 h prior to transfection into 24-well or 6-well plates or 6 cm dishes. Hsa-miR-143-5p (Applied Biosystems), hsa-miR-143-3p (Applied Biosystems) or a miRNA mimic control (Applied Biosystems) was transfected with Lipofectamine 2000 (Invitrogen) at a final concentration of 50 nmol/L. The sequences of the mature miR-143-5p and miR-143-3p used in this study were GGUGCAGUGCUGCAUCUCUGGU and UGAGAUGAAGCACUGUAGCUC, respectively. The cells were harvested at 24 h (for RNA extraction), 48 h (for protein extraction) or 72 h (for apoptosis assays).

Cell viability assays

An Alamar blue assay was used to measure cell proliferation. This assay is based on the quantitative metabolic conversion of blue, non-fluorescent resazurin to pink, fluorescent resorufin by living cells. After 72 h of incubation, an Alamar blue (Invitrogen) stock solution was aseptically added to the wells to equal to 10% of the total incubation volume. The resazurin reduction in the cultures was determined after a 2-6 h incubation with

Alamar blue by measuring the absorbances at 530-nm and 590-nm wavelengths on a Synergy HT Multi-Mode Microplate Reader (Bio-tek Instruments).

Apoptosis assay

Following maintenance in culture, the cells were harvested and stained with phycoerythrin-conjugated Annexin V according to the manufacturer's instructions (BD Biosciences). The cells were then analyzed on a FACSCalibur flow cytometer (BD Biosciences). The cells were considered viable if double negative, early apoptotic if positive for Annexin V alone and necrotic or late apoptotic if double positive.

MiRNA target prediction

RNA22 (http://cbcsrv.watson.ibm.com/rna22.html) was used to identify miRNA-target sites in the 3'-UTR of COX-2 mRNA and the corresponding RNA/RNA complexes and folding energies.

Western blot

Cells were lysed with Radio Immunoprecipitation Assay buffer (Sigma-Aldrich), and the total protein concentration was determined with a Bio-Rad Protein Assay (Bio-Rad). Proteins (40 μg) were separated by 10% SDS/PAGE and electrotransferred onto nitrocellulose membranes. The membranes were then incubated overnight with a COX-2 (Cell Signaling) or poly (ADP-ribose) polymerase (PARP) primary antibody (Cell Signaling) at 4 ℃, and subsequently incubated with an HRP-conjugated anti-rabbit secondary antibody (Bio-Rad) for 1 h at room temperature. Protein bands were detected with the Western Blotting Luminol Reagent (Santa Cruz Biotechnology).

Reverse transcription and real-time PCR to quantify COX-2 mRNA

Total RNA was extracted with TRIzol (Invitrogen). DNase I (Amplification Grade, Invitrogen) and the SuperScript First-Strand Synthesis System for reverse transcription-PCR (RT-PCR) (Invitrogen) were used for cDNA preparation. Primers and probes were ordered from IDT Inc. The following primers and probes were used: COX-2: Primer-F: 5'-CAAATCCTTGCTGTTCCCACCCAT-3', Primer-R: 5'-GTGCACTGTGTTTGGAGTGGGTTT-3', Probe: 5'-AAGTGCGATTGTACCCGGACAGGATT-3', β-GUS: Primer-F: 5'-CTCATTTGGAATTTTGCCGATT-3', Primer-R: 5'-CCGAGTGAAGATCCCCTTTTTA-3', Probe: 5'-TGAACAGTCACCGACGAGAGTGCTGG-3'. The reactions were incubated in a 96-well plate at 95 ℃ for 12 min, followed by 40 cycles of 95 ℃ for 30 s, 60 ℃ for 1 min, and 72 ℃ for 45 s. All reactions were performed in triplicate.

Luciferase reporter assay

The human COX-2 3'-UTR was amplified and cloned into the XbaI site of the pGL3-control vector (Promega, United States), downstream of the luciferase gene, to generate the plasmid pGL3-WT-COX2-3'-UTR. pGL3-MUT-5p-COX2-3'-UTR was generated from pGL3-WT-COX2-3'-UTR by deleting the "ACTGTAC" binding site for miR-143-5p. For the luciferase reporter assay, cells were cotransfected with the luciferase reporter vectors and miR-143-5p, miR-143-3p or a miRNA mimic control, using Lipofectamine 2000. A β-actin promoter Renilla luciferase reporter was used for normalization. After 48 h, luciferase activity was analyzed by the Dual-Glo Luciferase Assay System (Promega), according to the manufacturer's protocols.

Statistical analysis

Experimental results were assessed for significance with one-tailed unpaired *t* tests. A *P* value less than 0.05 was considered significant.

RESULTS

MiR-143-5p and miR-143-3p expression is reduced in gastric cancer cells

Using real-time PCR to quantify mature miR-143-5p and miR-143-3p in five human gastric cancer cell lines and a normal gastric epithelium cell line, we found that both the miR-143-5p and miR-143-3p expression levels were markedly reduced in gastric cancer cells ($P < 0.01$; Figure 1A and B). Western blot analysis indicated that COX-2 protein expression was increased in the five human gastric cancer cell lines (Figure 1C); this expression was inversely correlated with the miR-143 levels.

MiR-143 decreases viability and increases apoptosis in gastric cancer cells

The reduced miR-143 expression in gastric cancer suggests that this miRNA could have anti-proliferative effects. To test this hypothesis, we evaluated the effects of transient transfection with miR-143-5p and miR-143-3p in MKN-1 and BGC-823 gastric cancer cell lines. The Alamar Blue assay, a redox assay similar to the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, showed significant decreases in gastric cancer cell viability following the transfection of either miR-143-5p or miR-143-3p. The decrease in viability after miR-143-5p transfection was greater than that after miR-143-3p transfection ($P < 0.05$; Figure 2A). Additionally, cell counts were significantly decreased after transfection with either miR-143-5p or miR-143-3p ($P < 0.05$; Figure 2B). Consistent with the results of the Alamar Blue assay, miR-143-5p showed a stronger inhibitory effect than miR-143-3p.

An apoptosis assay after transfection with miR-143-5p or miR-143-3p indicated a marked increase in cell apoptosis. Cells transfected with miR-143-5p had a significantly higher apoptosis rate than those transfected with miR-143-3p ($P < 0.05$; Figure 2C and D). Furthermore, both miR-143-5p and miR-143-3p transfection were accompanied by increased levels of cleaved PARP, a product of apoptosis (Figure 2E).

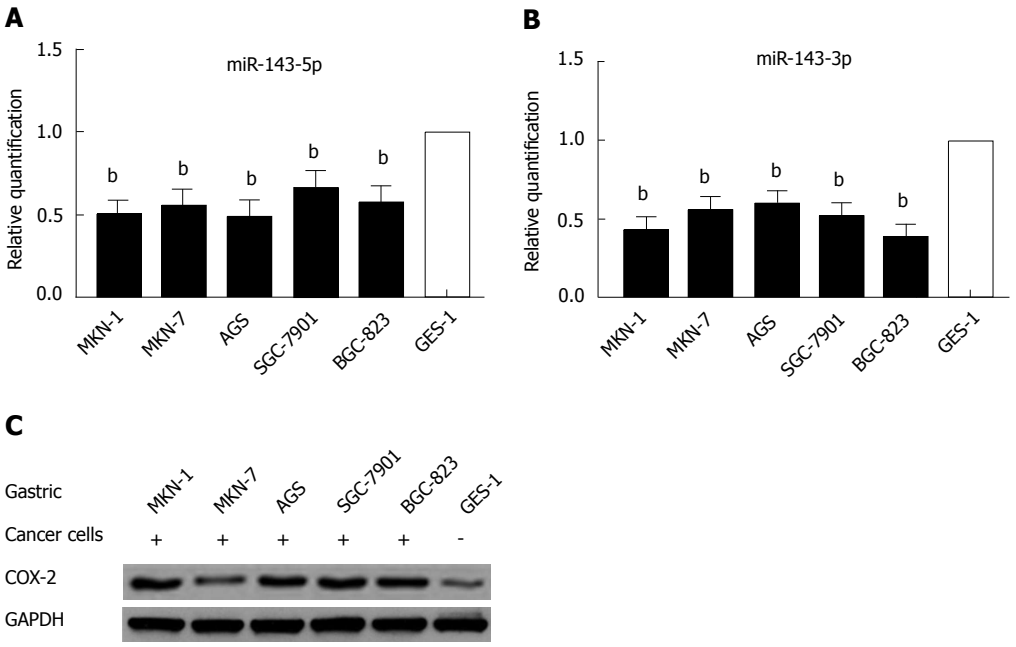


Figure 1 MicroRNA-143 expression is downregulated in gastric cancer cell lines. A: Quantitative real-time polymerase chain reaction analysis was performed in five gastric cell lines and a normal gastric epithelium cell line (GES-1). Mature microRNA-143-5p (miR-143-5p) expression levels were significantly downregulated in gastric cancer cells, compared to normal gastric epithelium cells ($^bP < 0.01$). The mean value from the GES-1 cell line was normalized to 1; B: Mature miR-143-3p expression levels were significantly downregulated in gastric cancer cells, compared to normal gastric epithelium cells ($^bP < 0.01$); C: Western blot analysis showed that cyclooxygenase-2 protein expression in the five human gastric cancer cell lines inversely correlated with the miR-143 levels. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; COX-2: Cyclooxygenase-2.

MiR-143 directly inhibits COX-2 expression via its 3'-UTR

A bioinformatics analysis, conducted with RNA22, indicated that the 3'-UTR of the human COX-2 mRNA (NM_000963) harbors a putative miR-143-5p binding site (nucleotides 3515-3536) (Figure 3A) but has no miR-143-3p binding site. To assess the inhibitory effects of miR-143 on COX-2, MKN-1 and BGC-823 cells were transfected with miR-143-5p, miR-143-3p or a control. Western blot analysis revealed a profound decrease in the COX-2 protein level after transfection with miR-143-5p but not miR-143-3p (Figure 3B). Consistent with transcriptional inhibition by miRNA, we noted decreased COX-2 mRNA levels after miR-143-5p transfection ($P < 0.05$; Figure 3C).

To confirm the direct action of miR-143 on the COX-2 3'-UTR, transient transfection experiments were performed with the 3'-UTR of COX-2, containing mutated or non-mutated putative miR-143-5p matching sites, downstream of the luciferase open reading frame. Transfection with miR-143-5p inhibited the normalized activity of the COX-2 3'-UTR reporter by 57% ($P < 0.01$), whereas MiR-143-3p had no effect on this reporter activity (Figure 3D). In contrast, co-transfection of the mutant reporter plasmid with either miR-143-5p or miR-143-3p had no effect on luciferase activity in the transfected cells (Figure 3E). These results demonstrated that only miR-143-5p bound to the seed sequence present in the 3'-UTR of human COX-2 mRNA to inhibit COX-2 expression.

DISCUSSION

In the present study, the expression levels of both miR-143-5p and miR-143-3p were found to be downregulated in gastric cancer. This finding is consistent with reports from other research groups[22,23]. In a recent report, the authors used real-time RT-PCR and chip assays to analyze 70 paired samples of gastric cancers and benign tissues[23]. The authors found that miR-143 was among the most strongly downregulated miRNAs in gastric cancers, compared to benign tissues. The miR-143 expression level was associated with gastric cancer progression and was more significantly reduced in stage Ⅳ cancers, compared with stage Ⅰ and Ⅱ cancers. Consistent with our observations, a study by Takagi *et al*[22] also indicated that miR-143 was downregulated in gastric cancer cell lines and that transfection with miR-143-3p inhibited the gastric cancer cell viability by targeting ERK5 and AKT. However, the function of miR-143-5p has never been investigated in gastric cancer.

In the miRNA biogenesis pathway, long primary transcripts (pre-miRNAs) that have been transcribed from the genome are processed by the cellular RNase enzyme Ⅲ Drosha into 60-110-nt structures called precursor miRNAs (pre-miRNA)[30]. Pre-miRNAs are cleaved by the RNase Ⅲ enzyme Dicer-1 to produce short, imperfect, double-stranded miRNA duplexes that are subsequently unwound by helicases to create mature miRNAs. In some cases, two mature miRNAs can be excised from

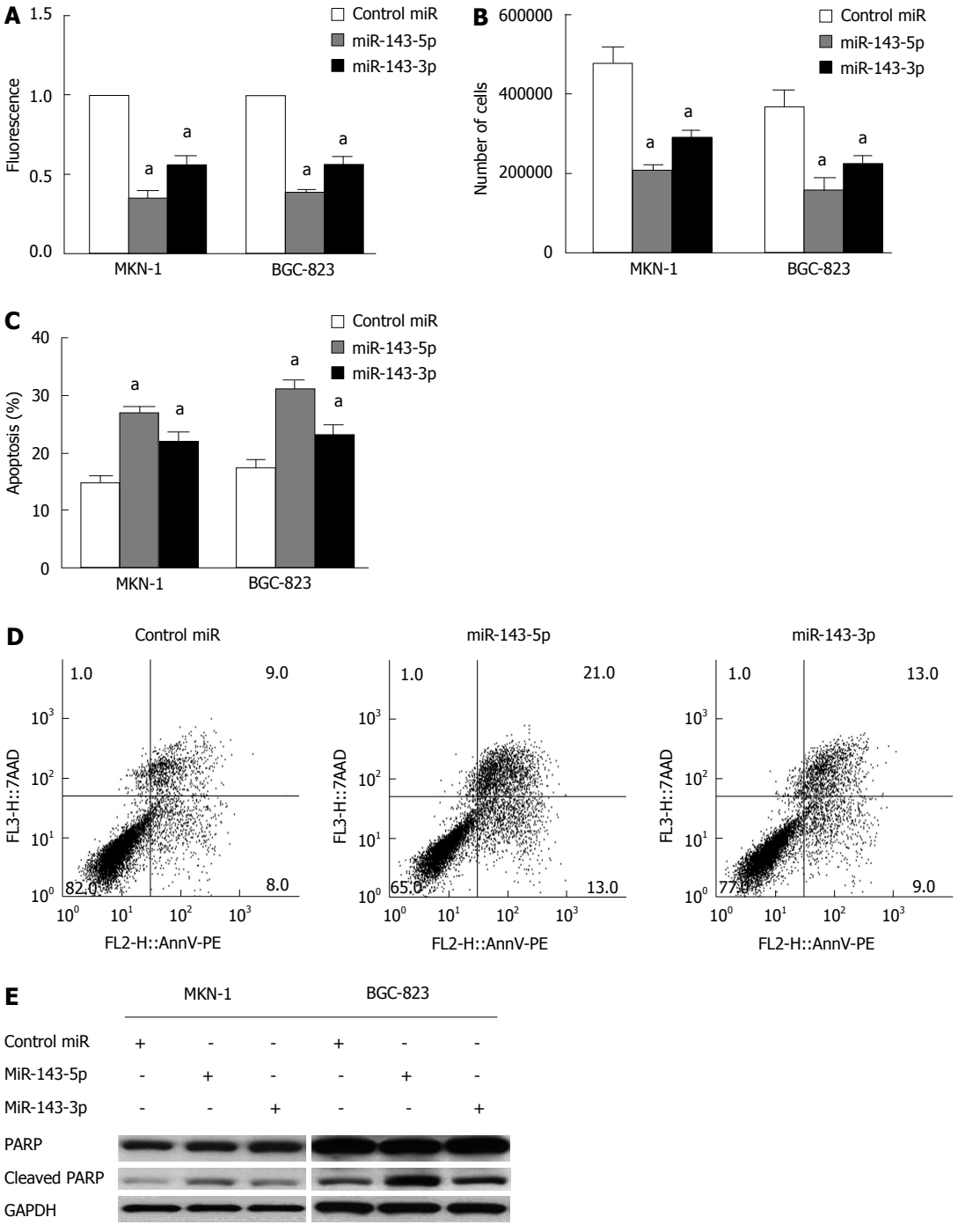


Figure 2 Transfection with microRNA-143 inhibits gastric cancer cell viability and induces apoptosis. A: An Alamar Blue assay was performed 3 d after transfection with microRNA-143-5p (miR-143-5p) or miR-143-3p to measure the viability of MKN-1 and BGC-823 gastric cancer cells. The results showed significant decreases in cell viability following transfection with either miR-143-5p or miR-143-3p ($^{a}P < 0.05$); this decrease was greater after miR-143-5p transfection than after miR-143-3p ($^{c}P < 0.05$); B: Cell counts performed 3 d after transfection into MKN-1 and BGC-823 gastric cancer cells showed decreased cell numbers after transfection with either miR-143-5p or miR-143-3p ($^{a}P < 0.05$). Consistent with the results of the Alamar Blue assay, the decrease in cell number after miR-143-5p transfection was greater than that after miR-143-3p transfection ($^{c}P < 0.05$); C and D: An Annexin V/PE cell apoptosis assay revealed increased apoptosis in gastric cancer cells after transfection with either miR-143-5p or miR-143-3p ($^{a}P < 0.05$). Cells transfected with miR-143-5p had a significantly higher apoptosis rate than those transfected with miR-143-3p ($^{c}P < 0.05$); E: Western blots of PARP protein in MKN-1 and BGC-823 cell lines at 3 d after transfection with miR-143-5p or miR-143-3p. GAPDH was used as a control. The results showed increased expression of cleaved PARP after miR-143-5p and miR-143-3p transfection. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase. PARP: Poly (ADP-ribose) polymerase.

the same stem-loop pre-miRNA[31]. These "5p" and "3p" miRNAs, although generated from a single primary transcript, have different sequences and therefore target different mRNAs. In humans, two different mature miRNA sequences are excised from opposite arms of the stem-loop pre-miR-143 to generate two different miRNAs, hsa-miR-143-5p and has-miR-143-3p. Despite nearly a decade of studies on the roles of miRNA in cancers, the

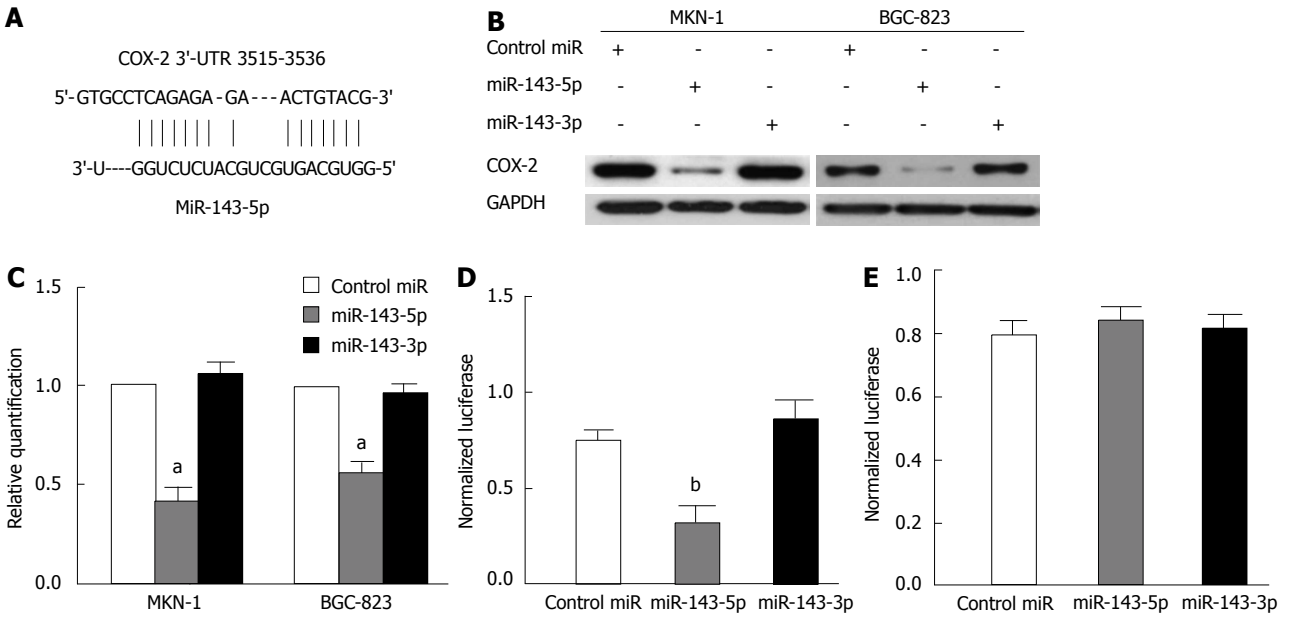


Figure 3 MicroRNA-143-5p directly inhibits cyclooxygenase-2 expression. A: Sites of miR-143-5p seed matches in the cyclooxygenase-2 (COX-2) 3'-untranslated region (3'-UTR) (nucleotides 3515-3536); B: Western blot of COX-2 protein in the MKN-1 and BGC-823 gastric cancer cell lines at 3 d after transfection with miR-143-5p, miR-143-3p or microRNA (miRNA) mimic control. GAPDH was used as a control. The results showed a profound decrease in COX-2 protein expression after transfection with miR-143-5p but not miR-143-3p; C: Real-time reverse transcription-polymerase chain reaction to determine COX-2 mRNA expression was performed 2 d after transfection with miR-143-5p, miR-143-3p or a control in MKN-1 and BGC-823. The mean expression in the control group was normalized to 1. Consistent with transcriptional inhibition by miRNA, the COX-2 mRNA level was reduced after miR-143-5p transfection ($^{a}P < 0.05$); D: Normalized activity of the wild-type COX-2 3'-UTR luciferase reporter in BGC-823 cells, 2 d after transfection with miR-143-5p, miR-143-3p or a control. The luciferase activity was significantly decreased by miR-143-5p ($^{b}P < 0.01$) but not miR-143-3p; E: Normalized activity of the mutant-type COX-2 3'-UTR luciferase reporter in BGC-823 cells, 2 d after transfection with miR-143-5p, miR-143-3p or a control. The results showed that cotransfection of the mutant reporter plasmid with miR-143-5p or miR-143-3p had no effect on luciferase activity in the transfected cells. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

comparative roles of strand-specific mature miRNAs that originate from the same stem-loop precursor (5p and 3p) have yet to be fully studied. Previously, a number of studies demonstrated that ectopic miR-143 expression inhibited cancer cells in various types of human tumors[4,6-8,16,20,22]. However, the role of miR-143 in gastric cancer has not been fully investigated, and differences between miR-143-5p and miR-143-3p have never been reported.

Our functional analysis of miR-143 expression in gastric cancer cell lines indicated tumor suppressor functions for both miR-143-5p and miR-143-3p. Our data revealed that the restoration of both miR-143-5p and miR-143-3p expression suppressed cell proliferation and promoted apoptosis in gastric cancer cells. The tumor-suppressive effect of miR-143-5p was stronger than that of miR-143-3p.

Further analysis demonstrated that only miR-143-5p directly binds to COX-2 mRNA. The luciferase reporter assay revealed that COX-2 contains a binding site for miR-143-5p but no binding site for miR-143-3p. A recent report also demonstrated different functions of miR-28-5p and miR-28-3p; specifically, miR-28-5p altered the expression of CCND1 and HOXB3, whereas miR-28-3p bound to NM23-H1 in colorectal cancer[32]. Our study provides further evidence that strand-specific "5p" and "3p" miRNAs could have different targets. This could explain why miR-143-5p is a stronger tumor suppressor in gastric cancer and suggests the existence of other potential targets of miR-143-3p. For example, other studies have revealed multiple targets for miR-143, such as KRAS[5,6], Bcl-2[7,8], PAI-1[9], MMP-13[11], DNMT3A[12], ELK1[13] and MYO6[10]. Further studies are needed to determine other targets of miR-143-5p and miR-143-3p in gastric cancer.

In conclusion, both miR-143-5p and miR-143-3p are downregulated in gastric cancer and function as anti-oncomirs. MiR-143-5p is more strongly tumor suppressive than miR-143-3p. Our data also indicate that COX-2 is a direct target of miR-143-5p but not of miR-143-3p. Further studies are needed to define the detailed mechanisms and identify more miR-143 targets.

COMMENTS

Background

MicroRNA-143 (miR-143) is considered a pivotal regulator of gene expression and directly targets multiple mRNAs that code for proteins involved in cell proliferation, differentiation, survival and apoptosis. It is downregulated in various human tumors and suppresses tumor growth in cancers of the urogenital system, digestive system, respiratory system and nervous system, as well as some sarcomas and B-cell malignancies. MiR-143 expression has also been reported to be downregulated in human gastric cancer tissues and cell lines, but the mechanism by which miR-143 regulates cancer cells is not fully clear.

Research frontiers

MiR-143 has been reported to be a tumor suppressor. However, the role of miR-143 in gastric cancer has not been fully investigated. The functional differences between miR-143-5p and miR-143-3p with regard to cancer have never been reported. In this study, the authors compared the tumor suppressive func-

tions of miR-143-5p and miR-143-3p and explored the associated underlying mechanism.

Innovations and breakthroughs

This functional analysis of miR-143 expression in gastric cancer cell lines indicated that both miR-143-5p and miR-143-3p act as tumor suppressors. The restoration of either miR-143-5p or miR-143-3p suppressed cell proliferation and promoted apoptosis in gastric cancer cells. The tumor-suppressive effect of miR-143-5p was stronger than that of miR-143-3p. Further analysis demonstrated that only miR-143-5p directly bound to the cyclooxygenase-2 (COX-2) mRNA. The luciferase reporter assay revealed that COX-2 contained a binding site for miR-143-5p but not for miR-143-3p. Western blot showed a profound decrease in COX-2 protein expression after transfection with miR-143-5p but not with miR-143-3p. The data in this article indicate that COX-2 is a direct target of miR-143-5p but not of miR-143-3p.

Applications

Both miR-143-5p and miR-143-3p function as anti-oncomirs and are downregulated in gastric cancers. MiR-143-5p is more strongly tumor suppressive than miR-143-3p and could be a potential gastric cancer therapeutic target.

Terminology

MiR-143 is considered a pivotal regulator of gene expression because it directly targets multiple mRNAs that code for proteins involved in cell proliferation, differentiation, survival and apoptosis. It is downregulated in various human tumors and suppresses tumor growth in cancers from the urogenital, digestive, respiratory and nervous system, as well as B-cell malignancies.

Peer review

The authors examined the effects and mechanisms of miR-143 subtypes on gastric cancer cell lines. They examined the effects of these oncomirs on growth, apoptosis and COX-2 activity. For the most part, the paper is straightforward and well written. The experiments are well described and the results are clearly presented.

REFERENCES

1 **Baek D**, Villén J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of microRNAs on protein output. *Nature* 2008; **455**: 64-71 [PMID: 18668037 DOI: 10.1038/nature07242]

2 **He L**, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y, Xue W, Zender L, Magnus J, Ridzon D, Jackson AL, Linsley PS, Chen C, Lowe SW, Cleary MA, Hannon GJ. A microRNA component of the p53 tumour suppressor network. *Nature* 2007; **447**: 1130-1134 [PMID: 17554337 DOI: 10.1038/nature05939]

3 **Lu J**, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. *Nature* 2005; **435**: 834-838 [PMID: 15944708 DOI: 10.1038/nature03702]

4 **Song T**, Zhang X, Wang C, Wu Y, Dong J, Gao J, Cai W, Hong B. Expression of miR-143 reduces growth and migration of human bladder carcinoma cells by targeting cyclooxygenase-2. *Asian Pac J Cancer Prev* 2011; **12**: 929-933 [PMID: 21790228]

5 **Chen X**, Guo X, Zhang H, Xiang Y, Chen J, Yin Y, Cai X, Wang K, Wang G, Ba Y, Zhu L, Wang J, Yang R, Zhang Y, Ren Z, Zen K, Zhang J, Zhang CY. Role of miR-143 targeting KRAS in colorectal tumorigenesis. *Oncogene* 2009; **28**: 1385-1392 [PMID: 19137007 DOI: 10.1038/onc.2008.474]

6 **Xu B**, Niu X, Zhang X, Tao J, Wu D, Wang Z, Li P, Zhang W, Wu H, Feng N, Wang Z, Hua L, Wang X. miR-143 decreases prostate cancer cells proliferation and migration and enhances their sensitivity to docetaxel through suppression of KRAS. *Mol Cell Biochem* 2011; **350**: 207-213 [PMID: 21197560 DOI: 10.1007/s11010-010-0700-6]

7 **Liu L**, Yu X, Guo X, Tian Z, Su M, Long Y, Huang C, Zhou F, Liu M, Wu X, Wang X. miR-143 is downregulated in cervical cancer and promotes apoptosis and inhibits tumor formation by targeting Bcl-2. *Mol Med Report* 2012; **5**: 753-760 [PMID: 22160209 DOI: 10.3892/mmr.2011.696]

8 **Zhang H**, Cai X, Wang Y, Tang H, Tong D, Ji F. microRNA-143, down-regulated in osteosarcoma, promotes apoptosis and suppresses tumorigenicity by targeting Bcl-2. *Oncol Rep* 2010; **24**: 1363-1369 [PMID: 20878132]

9 **Villadsen SB**, Bramsen JB, Ostenfeld MS, Wiklund ED, Fristrup N, Gao S, Hansen TB, Jensen TI, Borre M, Ørntoft TF, Dyrskjøt L, Kjems J. The miR-143/-145 cluster regulates plasminogen activator inhibitor-1 in bladder cancer. *Br J Cancer* 2012; **106**: 366-374 [PMID: 22108519 DOI: 10.1038/bjc.2011.520]

10 **Szczyrba J**, Löprich E, Wach S, Jung V, Unteregger G, Barth S, Grobholz R, Wieland W, Stöhr R, Hartmann A, Wullich B, Grässer F. The microRNA profile of prostate carcinoma obtained by deep sequencing. *Mol Cancer Res* 2010; **8**: 529-538 [PMID: 20353999 DOI: 10.1158/1541-7786.MCR-09-0443]

11 **Osaki M**, Takeshita F, Sugimoto Y, Kosaka N, Yamamoto Y, Yoshioka Y, Kobayashi E, Yamada T, Kawai A, Inoue T, Ito H, Oshimura M, Ochiya T. MicroRNA-143 regulates human osteosarcoma metastasis by regulating matrix metalloprotease-13 expression. *Mol Ther* 2011; **19**: 1123-1130 [PMID: 21427707 DOI: 10.1038/mt.2011.53]

12 **Ng EK**, Tsang WP, Ng SS, Jin HC, Yu J, Li JJ, Röcken C, Ebert MP, Kwok TT, Sung JJ. MicroRNA-143 targets DNA methyltransferases 3A in colorectal cancer. *Br J Cancer* 2009; **101**: 699-706 [PMID: 19638978 DOI: 10.1038/sj.bjc.6605195]

13 **Cordes KR**, Sheehy NT, White MP, Berry EC, Morton SU, Muth AN, Lee TH, Miano JM, Ivey KN, Srivastava D. miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. *Nature* 2009; **460**: 705-710 [PMID: 19578358]

14 **Chang KH**, Miller N, Kheirelseid EA, Lemetre C, Ball GR, Smith MJ, Regan M, McAnena OJ, Kerin MJ. MicroRNA signature analysis in colorectal cancer: identification of expression profiles in stage II tumors associated with aggressive disease. *Int J Colorectal Dis* 2011; **26**: 1415-1422 [PMID: 21739196 DOI: 10.1007/s00384-011-1279-4]

15 **Wijnhoven BP**, Hussey DJ, Watson DI, Tsykin A, Smith CM, Michael MZ. MicroRNA profiling of Barrett's oesophagus and oesophageal adenocarcinoma. *Br J Surg* 2010; **97**: 853-861 [PMID: 20301167 DOI: 10.1002/bjs.7000]

16 **Wu BL**, Xu LY, Du ZP, Liao LD, Zhang HF, Huang Q, Fang GQ, Li EM. MiRNA profile in esophageal squamous cell carcinoma: downregulation of miR-143 and miR-145. *World J Gastroenterol* 2011; **17**: 79-88 [PMID: 21218087 DOI: 10.3748/wjg.v17.i1.79]

17 **Chen HC**, Chen GH, Chen YH, Liao WL, Liu CY, Chang KP, Chang YS, Chen SJ. MicroRNA deregulation and pathway alterations in nasopharyngeal carcinoma. *Br J Cancer* 2009; **100**: 1002-1011 [PMID: 19293812 DOI: 10.1038/sj.bjc.6604948]

18 **Gao W**, Yu Y, Cao H, Shen H, Li X, Pan S, Shu Y. Deregulated expression of miR-21, miR-143 and miR-181a in non small cell lung cancer is related to clinicopathologic characteristics or patient prognosis. *Biomed Pharmacother* 2010; **64**: 399-408 [PMID: 20363096 DOI: 10.1016/j.biopha.2010.01.018]

19 **Amaral FC**, Torres N, Saggioro F, Neder L, Machado HR, Silva WA, Moreira AC, Castro M. MicroRNAs differentially expressed in ACTH-secreting pituitary tumors. *J Clin Endocrinol Metab* 2009; **94**: 320-323 [PMID: 18840638 DOI: 10.1210/jc.2008-1451]

20 **Ugras S**, Brill E, Jacobsen A, Hafner M, Socci ND, Decarolis PL, Khanin R, O'Connor R, Mihailovic A, Taylor BS, Sheridan R, Gimble JM, Viale A, Crago A, Antonescu CR, Sander C, Tuschl T, Singer S. Small RNA sequencing and functional characterization reveals MicroRNA-143 tumor suppressor activity in liposarcoma. *Cancer Res* 2011; **71**: 5659-5669 [PMID: 21693658 DOI: 10.1158/0008-5472.CAN-11-0890]

21 **Akao Y**, Nakagawa Y, Kitade Y, Kinoshita T, Naoe T. Downregulation of microRNAs-143 and -145 in B-cell malignancies. *Cancer Sci* 2007; **98**: 1914-1920 [PMID: 17892514 DOI: 10.1111/j.1349-7006.2007.00618.x]

22 **Takagi T**, Iio A, Nakagawa Y, Naoe T, Tanigawa N, Akao Y. Decreased expression of microRNA-143 and -145 in human gastric cancers. *Oncology* 2009; **77**: 12-21 [PMID: 19439999

DOI: 10.1159/000218166]
23 **Li X**, Luo F, Li Q, Xu M, Feng D, Zhang G, Wu W. Identification of new aberrantly expressed miRNAs in intestinal-type gastric cancer and its clinical significance. *Oncol Rep* 2011; **26**: 1431-1439 [PMID: 21874264]
24 **Hoellen F**, Kelling K, Dittmer C, Diedrich K, Friedrich M, Thill M. Impact of cyclooxygenase-2 in breast cancer. *Anticancer Res* 2011; **31**: 4359-4367 [PMID: 22199301]
25 **Adhim Z**, Matsuoka T, Bito T, Shigemura K, Lee KM, Kawabata M, Fujisawa M, Nibu K, Shirakawa T. In vitro and in vivo inhibitory effect of three Cox-2 inhibitors and epithelial-to-mesenchymal transition in human bladder cancer cell lines. *Br J Cancer* 2011; **105**: 393-402 [PMID: 21750550 DOI: 10.1038/bjc.2011.262]
26 **Yin J**, Liu B, Li B, Liu Z, Xie X, Lv Z, Gao S, Guang J. The cyclooxygenase-2 inhibitor celecoxib attenuates hepatocellular carcinoma growth and c-Met expression in an orthotopic mouse model. *Oncol Res* 2011; **19**: 131-139 [PMID: 21473289 DOI: 10.3727/096504011X12935427587803]
27 **Zhong B**, Cai X, Chennamaneni S, Yi X, Liu L, Pink JJ, Dowlati A, Xu Y, Zhou A, Su B. From COX-2 inhibitor nimesulide to potent anti-cancer agent: synthesis, in vitro, in vivo and pharmacokinetic evaluation. *Eur J Med Chem* 2012; **47**: 432-444 [PMID: 22119125 DOI: 10.1016/j.ejmech.2011.11.012]
28 **Ma D**, Liu M, Wang AP, Yang H. Cycloxygenase-2 is essential for the survival and proliferation of gastric cancer cells. *Cell Biochem Biophys* 2011; **61**: 637-641 [PMID: 21830126 DOI: 10.1007/s12013-011-9249-6]
29 **Wu WK**, Cho CH, Lee CW, Fan D, Wu K, Yu J, Sung JJ. Dysregulation of cellular signaling in gastric cancer. *Cancer Lett* 2010; **295**: 144-153 [PMID: 20488613 DOI: 10.1016/j.canlet.2010.04.025]
30 **Lee Y**, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Rådmark O, Kim S, Kim VN. The nuclear RNase III Drosha initiates microRNA processing. *Nature* 2003; **425**: 415-419 [PMID: 14508493 DOI: 10.1038/nature01957]
31 **Griffiths-Jones S**, Grocock RJ, van Dongen S, Bateman A, Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res* 2006; **34**: D140-D144 [PMID: 16381832 DOI: 10.1093/nar/gkj112]
32 **Almeida MI**, Nicoloso MS, Zeng L, Ivan C, Spizzo R, Gafà R, Xiao L, Zhang X, Vannini I, Fanini F, Fabbri M, Lanza G, Reis RM, Zweidler-McKay PA, Calin GA. Strand-specific miR-28-5p and miR-28-3p have distinct effects in colorectal cancer cells. *Gastroenterology* 2012; **142**: 886-896.e9 [PMID: 22240480 DOI: 10.1053/j.gastro.2011.12.047]

P- Reviewers: Hahm KB, Kanda T, Raffaniello RD
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.v19.i43.7766

World J Gastroenterol 2013 November 21; 19(43): 7766-7771
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

PU.1-silenced dendritic cells prolong allograft survival in rats receiving intestinal transplantation

Xing-Wei Xu, Bo-Wen Ding, Chuan-Rong Zhu, Wu Ji, Jie-Shou Li

Xing-Wei Xu, Chuan-Rong Zhu, Wu Ji, Jie-Shou Li, Jinling Hospital, Research Institute of General Surgery, Nanjing University, School of Medicine, Nanjing 210002, Jiangsu Province, China
Bo-Wen Ding, Department of General Surgery, Xuzhou Hospital of Traditional Chinese Medicine, Xuzhou 221001, Jiangsu Province, China
Author contributions: Ji W designed the research and analyzed the data; Xu XW performed the research, analyzed the data and wrote the paper; Ding BW and Zhu CR performed the research; and Li JS guided and supervised the research.
Supported by A grant from the Natural Science Foundation of Jiangsu Province, China, No. BK2008237
Correspondence to: Wu Ji, MD, Professor, Jinling Hospital, Research Institute of General Surgery, Nanjing University, School of Medicine, No.305, East Zhongshan Road, Xuanwu District, Nanjing 210002, Jiangsu Province, China. jiwusky@126.com
Telephone: +86-25-80863334 Fax: +86-25-80863334
Received: July 20, 2013 Revised: September 5, 2013
Accepted: September 29, 2013
Published online: November 21, 2013

Abstract

AIM: To investigate the function of PU.1-silenced semi-mature dendritic cells (DCs) and the possibility of utilizing cell immunity in rat intestinal transplantation.

METHODS: DCs were isolated from the bone marrow of F344 rats and cultured using the adherent method. The *PU.1* gene was knocked down in DCs using small interfering RNAs (siRNAs) for 24 h, and the cells were then incubated with lipopolysaccharide for 48 h. The PU.1 siRNA that had the highest silencing efficiency was screened using reverse transcription-polymerase chain reaction and Western blot for further study. The tolerance capacity was analyzed and compared between PU.1-silenced DCs (siRNA PU.1 group), negative control-silenced DCs (siRNA NC group) and immature DCs (control group) both *in vitro* and *in vivo*.

CONCLUSION: Blocking expression of the *PU.1* gene *in vitro* led to a reduction in DC maturation and an increased tolerance capability. PU.1-silenced DCs expressed moderate levels of major histocompatibility complex (MHC)-Ⅱ and low levels of co-stimulatory molecules, and produced more interleukin (IL)-10, but less IL-12. Compared with the negative control group, surface molecules cluster of differentiation 80 (CD80), CD86 and MHC-Ⅱ in the siRNA PU.1 group were 27.0% ± 5.6%, 23.6% ± 4.8% and 36.8% ± 6.8%, respectively, and showed a significantly lower trend ($P < 0.05$). *In vivo* treatment of recipients with PU.1-silenced DCs injected before intestinal transplantation (siRNA PU.1 group), significantly prolonged allograft survival and resulted in better tissue histopathology compared with the siRNA NC group and control group. Mean survival time after transplantation was 14.3 ± 3.3 d in the siRNA PU.1 group ($P < 0.05$).

CONCLUSION: PU.1-silenced semi-mature DCs induced partial immune tolerance both *in vitro* and *in vivo*, which could be used as a new strategy to promote transplantation tolerance.

Key words: Dendritic cell; PU.1; Tolerance; Intestinal transplantation; Immune tolerance

Core tip: The inhibition of dendritic cells (DCs) maturation can promote their tolerogenicity in transplantation. PU.1 is a newly discovered transcription factor which is required for the regulation of dendritic cell maturation in all DCs subsets. We silenced the *PU.1* gene using siRNA and showed, for the first time, that PU.1-silenced DCs had immune tolerance. This may be a new strategy to prevent graft rejection following intestinal transplantation.

Xu XW, Ding BW, Zhu CR, J i W, Li JS. PU.1-silenced den-

dritic cells prolong allograft survival in rats receiving intestinal transplantation. *World J Gastroenterol* 2013; 19(43): 7766-7771 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7766.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7766

INTRODUCTION

Dendritic cells (DCs) are key antigen-presenting cells, which play an important role in regulating adaptive immune responses. Studies have shown that whether immune responses are induced or suppressed greatly depends on the degree of DC maturation and specific subsets[1,2]. Immature DCs, which express low levels of major histocompatibility complex (MHC-Ⅱ) and co-stimulatory molecules, such as cluster of differentiation 80 (CD80), CD86 and CD40, have a lower ability to capture antigens for presentation to specific T cells[3,4]. Therefore, various approaches have been explored to inhibit the maturation of DCs and to promote their tolerogenicity[5].

MicroRNA-155 has emerged as an important regulator in the immune system[6,7]. MicroRNA (mRNA)-155 knockout mice showed aberrant immune functions, such as defective B and T cell immunity, abnormal function of antigen-presenting cells, and a failure in the production of high-affinity Immunoglobulin G (IgG)1 antibodies[8,9]. These phenotypes are related to the impaired ability of mRNA-155 to target the E-twenty six transcription factor PU.1, which was first discovered to have multiple roles in hematopoiesis. PU.1 is an essential regulator of both cDC and pDC lineages[10,11], and can regulate numerous genes within the myeloid and lymphoid lineages[12]. Recent studies have shown that PU.1 can partially direct the important cytokine receptor Flt3 and play a critical role in DC development and function. Therefore, PU.1 is a major and critical regulator of DC maturation.

In this study, we silenced PU.1 expression in rat bone marrow DCs (BM cells) using small interference RNA (siRNA) molecules and stimulated the cells with lipopolysaccharide (LPS) to obtain semi-mature DCs. These semi-mature DCs were then used to determine whether they could induce tolerance and have an effect on intestinal transplantation in rats.

MATERIALS AND METHODS

Animals

F344 and Wistar rats (weighing 180-220 g) were purchased from the Vital River Corporation (Beijing, China) and kept under specific-pathogen-free conditions. Animal experiments and maintenance were approved and regulated by the Ethics Committee of Jinling Hospital (Nanjing, China).

In vitro generation of bone marrow-derived immature DCs

BM cells of F344 rats were used for DC generation following the method described by Lutz *et al*[13] and Yang *et al*[14]. Briefly, the femur and tibia were mechanically obtained, and the marrow cells were flushed out using phosphate-buffered saline (PBS). The obtained single cell suspensions were centrifuged, treated with 0.15 mol/L NH_4Cl for 5 min and washed twice. The harvested BM cells were cultured in six-well plates (density, 4×10^6/mL) in RPMI1640 with 5 ng/mL recombinant rat granulocyte-macrophage colony-stimulating factor and 5 ng/mL interleukin (IL)-4 (Peprotech, NJ, United States). Non-adherent granulocytes were removed after 48 h of culture. From day 3, half of the medium was replaced with fresh medium every other day. On day 7, non-adherent and loosely adherent cells were harvested and identified as immature DCs, which were ready for transfection, and the supernatants were used for cytokine detection.

Treatment of DCs

For *in vitro* studies, siRNAs targeting the *PU.1* gene were synthesized by Jima Corporation (Shanghai, China)[10,15]. The siRNAs were transiently transfected into the cells using Lipofectamine 2000 (Invitrogen, United States) for 24 h according to the manufacturer's instructions. The sequences of a PU.1-specific siRNA were: sense, 5'-AGCGAUCACUAUUGGGAUUTT-3'; and antisense, 5'-AAUCCCAAUAGUGAUCGCUTT-3'. The sequences of a negative control siRNA were: sense, 5'-UUCUCCGAACGUGUCACGUTT-3'; and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. Transfected DCs were cultured in the presence of 10 μg/mL LPS (Sigma-Aldrich, United States) for a further 48 h. Cells and supernatants were harvested for later use, and the cells were designated as PU.1-silenced-LPS DCs (siRNA PU.1 group), negative control-silenced-LPS DCs (siRNA NC group) or immature DCs (control group).

Real-time PCR

Total RNA was extracted from cells using Trizol (Invitrogen, United States). RNA (1 pg) was reverse transcribed using an oligo-(dT) primer and reverse transcriptase (Invitrogen). All the measurements were performed in triplicate for each sample and normalized to the β-actin gene. The primer sequences for PU.1 were: forward, 5'-GAGTTTGAGAACTTCCCTGAG-3'; and reverse, 5'-TGGTAGGTCATCTTCTTGCGG-3'. Primer sequences for β-actin were: forward, 5'-ATGGATGACGATATCGCT-3'; and reverse, 5'-ATGAGGTAGTCTGTCAGGT-3'[15].

Western blot

Cells were homogenized in RIPA lysis buffer and used for Western blot assays. Briefly, equal amounts of protein extracts were boiled in sodium dodecyl sulfate (SDS)-sample buffer for 5 min before being electrophoretically resolved on SDS polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). The membranes were blocked with 5% fat-free dried milk and bovine serum albumin dissolved in Tris Buffered Saline with

Tween-20 for 1 h at room temperature and incubated overnight at 4 ℃ with antibodies raised against PU.1 (Santa Cruz, United States) according to the manufacturer's instructions. Binding of these primary antibodies was visualized with goat anti-rabbit secondary antibodies (1:2000 dilution; Santa Cruz, Texas, United States). Finally, the membranes were washed and an emitter-coupled logic signal detection kit was used (Amersham, IL, United States) for signal detection.

Flow cytometric analysis

The following antibodies were purchased from eBioscience Corporation (CA, United States): phycoerythrin (PE)-coupled anti-CD86, PE-coupled anti-CD80 and fluorescein isothiocyanate-coupled anti-MHC-Ⅱ. OX62-Alexa Fluor was obtained from BioLegend (CA, United States). After 7 d of cultivation, the prepared cells mentioned above were stained using the above antibodies at 4 ℃ for 30 min in PBS containing 0.1% sodium azide. Phenotypic analysis of DCs was performed on a fluorescence activated cell sorter Calibur flow cytometer equipped with Cell Quest (Becton Dickinson, New Jersey, United States).

Purification of T cells and mixed lymphocyte reaction

T cells (2×10^5) purified from rat splenocytes (responder cells) were plated with immature DCs, PU.1-silenced DCs or negative control-silenced DCs (stimulator cells) at varying ratios. Cells were cultured for 3 d and pulsed with 1 μCi of [^{3}H] thymidine (PerkinElmer, Woodbridge, United States) for the final 18 h. The cells were subsequently harvested onto glass fiber filters, and incorporated radioactivity was quantified using a liquid scintillation counter.

Detection of IL-12p70 and IL-10

The supernatants from each group as described above were collected and the cytokines IL-12p70 and IL-10 were measured by enzyme-linked immunosorbent assay according to the manufacturer's instructions (R and D Systems, Minneapolis, United States).

Intestinal transplantation and treatment

Recipient Wistar rats, six in each group, were treated with PU.1-silenced DCs, negative control-silenced-LPS DCs or immature DCs from donor F344 rats (1×10^6 cells), seven days prior to intestinal transplantation *via* tail vein injection. Heterotopic intestinal transplantation was performed using the technique described by Zhang *et al*[16]. The state of intestinal health/rejection was monitored and evaluated daily by examining the color of the graft and secretions from the stoma. Recipient rats that died within three days were regarded as technical failures and excluded from further analysis. The allografts were collected from a location 5 cm from the origin of the jejunum on day 5 after transplantation. Tissues from the three groups were sectioned and subjected to HE staining to evaluate morphologic changes.

Statistical analysis

Data were reported as mean ± SD. One-way analysis of variance was used for data analysis within groups. *P* values less than 0.05 were considered significant.

RESULTS

In vitro silencing of PU.1 with siRNAs

In order to silence the *PU.1* gene in DCs, we constructed three pre-siRNA vectors targeting PU.1 and one vector carrying a negative control siRNA. We incubated synthetic siRNAs with DCs which were induced with GM-CSF and IL-4 for 7 d to validate the efficiency of gene silencing. The most efficient plasmid to silence PU.1 was selected by assaying the PU.1 mRNA and protein expression. Forty-eight hours after transfection, PU.1 expression in the siRNA PU.1 group was reduced by approximately 85% at the protein level compared with the siRNA NC group (Figure 1A).

Characteristics of semi-mature DCs and expression of cytokines

Similar to the characteristics of mature DCs, the DCs in the siRNA NC group also expressed high levels of MHC class Ⅱ and co-stimulatory molecules. However, in the siRNA PU.1 group, the DCs were semi-mature, with the expression of CD80, CD86 and MHC-Ⅱ (27.0% ± 5.6%, 23.6% ± 4.8% and 36.8% ± 6.8%, respectively) significantly lower than that in the siRNA NC group (74.0% ± 9.4%, 76.5% ± 8.7% and 87.8% ± 11.3%, respectively) (Figure 1B, $P < 0.05$). The ability of DCs in the three groups to produce cytokines in cell culture supernatants was also determined, and an opposite trend was noted between IL-12p70 and IL-10 production ($P < 0.05$) (Figure 1C). These data indicate that the PU.1 silencing partially inhibits DC maturation.

Impaired ability of semi-mature DCs to stimulate T cell proliferation

Purification of T cells and MLR analysis were performed to observe the *in vitro* activity of DCs. The proliferation of Wistar rat splenic T cells in a primary mixed lymphocyte reaction (MLR) in response to stimulation with DCs in the siRNA PU.1 group was significantly reduced compared with that in the siRNA NC group ($P < 0.05$, Figure 2), suggesting that PU.1-silenced DCs have an impaired capacity to stimulate T cell proliferation.

Treatment with PU.1-silenced DCs prolongs allograft survival

Since the results of the *in vitro* study showed that silencing of PU.1 reduced DC maturation and inhibited allogeneic T cell proliferation, we postulated that knockdown of this key transcription factor might prevent graft rejection. To determine this, we treated Wistar recipients with different groups of DCs 7 d before performing intestinal transplantation. While recipient survival was short in the

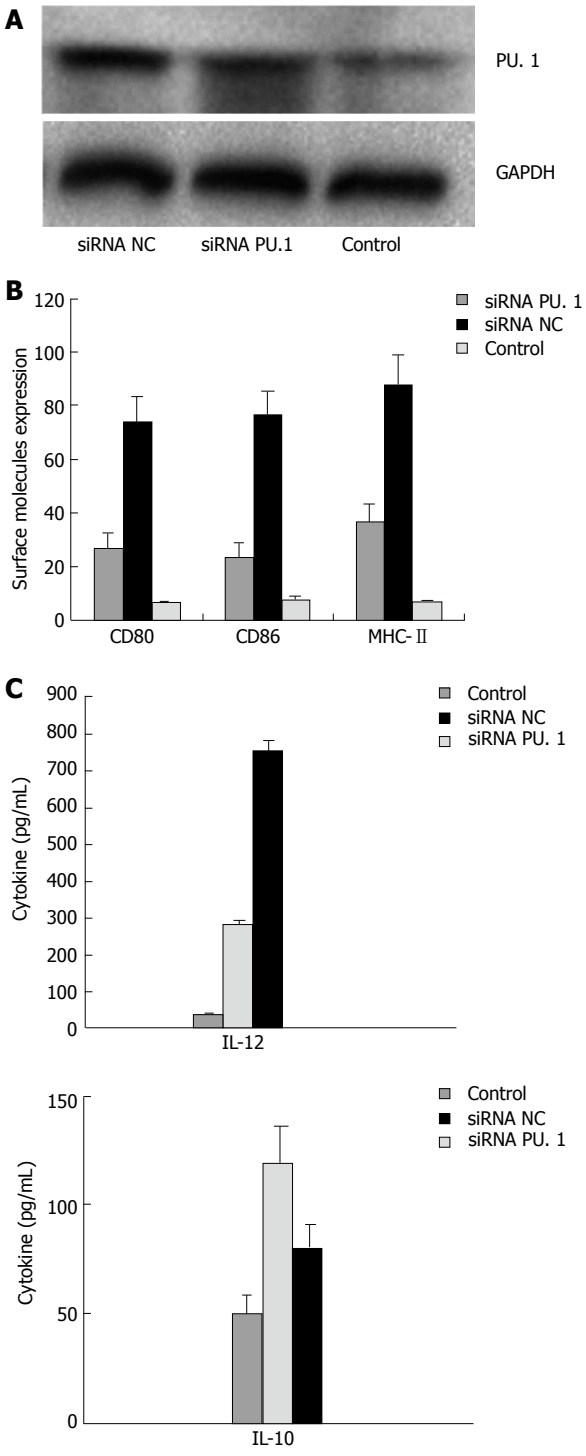


Figure 1 Identification of PU.1-silenced dendritic cells *in vitro*. A: Analyses of *PU.1* gene expression by Western blot; B: Flow cytometry analysis of CD80, CD86 and major histocompatibility complex (MHC)-Ⅱ in dendritic cells; C: Analyses of cell culture supernatants by enzyme-linked immunosorbent assay. CD: Cluster of differentiation; GAPDH: Reduced glyceraldehyde-phosphate dehydrogenase.

siRNA NC group (7.8 ± 1.5 d, $n = 6$, $P < 0.05$) and the control group (8.0 ± 2.5 d, $n = 6$, $P < 0.05$), the infusion of siRNA PU.1 DCs significantly prolonged survival (14.3 ± 3.3 d). Consistent with our surmise, morphological features of acute rejection were prominent in the siRNA NC group and in the control group. Histological examination

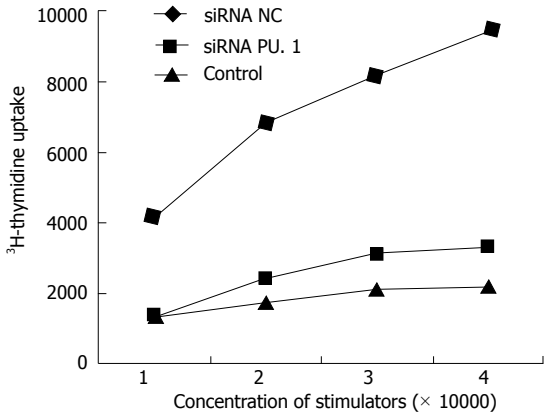


Figure 2 PU.1-silenced dendritic cells suppress allogeneic T cell proliferation. Bone marrow dendritic cells (DCs) were cultured and transfected with PU.1 siRNA as described. After that, three groups of DCs were collected and co-cultured with allogeneic T cells in a 96-well plate at various ratios as indicated. [^{3}H] was added 48 h after coculture, and its incorporation was measured as an indicator of T cell proliferation ($P < 0.05$ siRNA PU.1 *vs* siRNA NC group).

showed different degrees of lymphocyte infiltration and villous edema. In contrast, PU.1-silenced DCs delayed and reduced the immune response and injury, with mild lymphocyte infiltration and reduced inflammation observed in the allograft intestine (Figure 3).

DISCUSSION

Recently, the role of innate immunity in shaping the adaptive response has been focused in transplantation research, and studies have shown that the infusion of donor immature DCs can prolong graft survival after organ transplantation[17,18], mainly because they are capable of inducing tolerance by inducing T cell anergy or apoptosis[19,20]. Immature DCs express low levels of MHC Ⅱ and co-stimulatory molecules and fail to elicit naïve T cells to modulate the adaptive immune response. However, they are not stable *in vivo* and can easily be stimulated to transform into mature DCs through several signaling pathways[21], which limits their preservation and utilization. Recent studies show that by controlling ambient conditions *in vitro*, semi-mature DCs are obtained from immature DCs following LPS stimulation. These cells are phenotypically stable and hard to differentiate or mature. Yang *et al*[14] found that silencing of MyD88, a proximal component of nuclear factor-kappaB (NF-κB) signaling, affected the maturation of immature DCs by increasing the secretion of IL-10 and decreasing the secretion of IL-12p70. The NF-κB signaling pathway plays a critical role in DC maturation, and IL-10 is regarded as an immunosuppressive cytokine which can downregulate the synthesis of a broad range of inflammatory cytokines and inhibit allogeneic T cell proliferation[22]. Therefore, the silencing of key factors involved in DC maturation may lead to a stunted capacity to prime the immune response and better stability[23,24]. As PU.1 is highly expressed and plays a critical role in DC maturation, suppression of this gene may result in interruption of DC maturation.

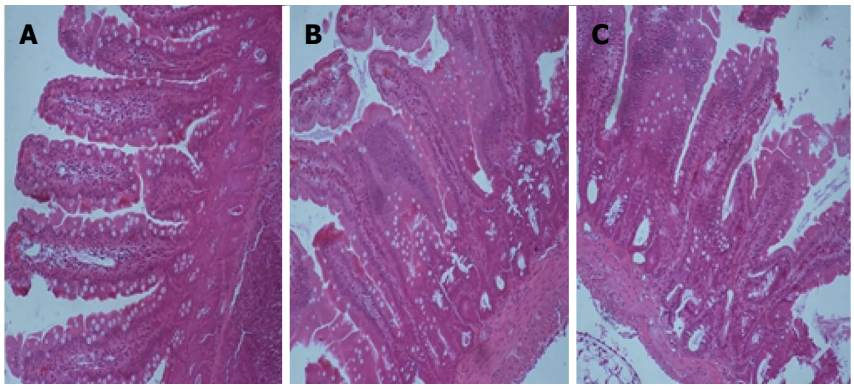


Figure 3 Histopathology of intestinal allograft from recipient rats. Treatment with PU.1 small interference RNA (siRNA) prevents allograft rejection and is associated with mild lymphocyte infiltration and villous damage. Samples from the three groups were compared (magnification × 40, scale bar 20 μm). A: The siRNA PU.1 group; B: The siRNA NC group; C: The control group.

Unsurprisingly, we found that PU.1-silenced semi-mature DCs[25] had a better effect in reducing the inflammatory response than immature DCs in an intestinal allograft model.

It is difficult to perform rat intestinal transplantation due to complex microvascular techniques and high mortality. Many animals died of immune rejection within several days. Although immature DCs express low levels of MHC II and determine tolerogenicity, current evidence for the application of immature DCs in rodent transplantation models is equivocal. In our experiment, rat survival, cytokine production and intestinal histological changes were evaluated to test the immunosuppressive function of semi-mature DCs. We found that acute rejection was significantly alleviated on day 5 compared to the controls, along with prolonged survival time and better condition in these rats. Rats in the siRNA PU.1 group showed slowed neointima formation and reduced inflammation and fibrosis in the allograft intestine. These results can be explained by increased secretion of IL-10 and decreased IL-12p70. The increase in IL-10 may play a crucial role in mediating the functions of semi-mature DCs. The surface expression of co-stimulatory molecules, such as CD86, CD80 and CD40, also showed a reduced trend, which is consistent with the results of T-cell proliferation. Thus, our experiments demonstrated that *PU.1* gene silencing induced partial tolerance in this animal transplantation model.

However, we do not know whether the number of semi-mature DCs fluctuated or changed in recipient rats, and whether these DCs reduced the number and maturation of native DCs at the time of small bowel transplantation. More *in vivo* studies in both donors and recipients are required to identify the mechanism related to better graft survival.

In conclusion, we have provided evidence that silencing of the *PU.1* gene can impair DC maturation, inhibit allogeneic T cell proliferation, and induce immunosuppressive activity. Since PU.1 silencing can prolong intestinal transplant survival in rats, it may be used as a new strategy and viable therapeutic option to prevent graft rejection following intestinal transplantation.

ACKNOWLEDGMENTS

We thank Yao Nie, Jian Wang, Bo Shen, Chun Tang, Qin He, Chen-Yang Wang for their encouragement and technical support in the laboratory tests; and Yu-Qin Xu for managing the supplies of the animal room for the research in Jinling Hospital, Nanjing, China.

COMMENTS

Background

As key antigen-presenting cells, whether dendritic cells (DCs) induce or suppress immune responses greatly depends on the degree of DC maturation and specific subsets. MicroRNA-155 has emerged as an important regulator in the immune system, and the transcription factor PU.1 is a direct target of miR-155, which has recently been found to play multiple critical roles in DC maturation and function.

Research frontiers

PU.1 is a major and critical regulator of DC maturation. However, the mechanism of action of PU.1 in DC maturation and tolerogenicity has not been unequivocally addressed. In this study, the authors silenced the *PU.1* gene using siRNA and demonstrated that PU.1 silencing impaired DC maturation and inhibited allogeneic T cell proliferation. PU.1-silenced DCs also prolonged intestinal transplant survival and improved the general state of the graft.

Innovations and breakthroughs

Recent reports have highlighted the important role of PU.1 in DC maturation and function. This is the first study to report that PU.1-silenced DCs can induce tolerogenicity, which can be applied in rat intestinal transplantation. Furthermore, the authors' *in vitro* studies suggest that inhibiting a key factor in a given signaling pathway is an effective way of inducing DC tolerogenicity.

Applications

The authors' finding that PU.1-silenced dendritic cells can prolong intestinal transplant survival in rats suggest that PU.1 silencing may be used as a new strategy and viable therapeutic option to prevent graft rejection following intestinal transplantation.

Terminology

PU.1 is an EPS transcription factor which was first discovered to play multiple roles in hematopoiesis. Recent studies have shown that PU.1 is an essential regulator of both cDC and pDC lineages and can regulate numerous genes within the myeloid and lymphoid lineages. Such a mechanism is thought to be crucial in inducing stable immature DCs. Not surprisingly, the DCs the authors cultivated in the study showed tolerogenicity both *in vivo* and *in vitro*.

Peer review

The authors examined the surface molecule expression of PU.1-silenced DCs, T cell proliferation and cytokines of cell culture supernatants. The cells were injected to recipient rats to test the immunogenicity *in vivo*. It revealed that they induced immunosuppressive activity; and the increased interleukin-10 expression may play a crucial role in the semi-mature DC functions and induce immunogenicity. The results are interesting and may supplement molecular mechanism of PU.1.

REFERENCES

1 **Santiago-Schwarz F**. Positive and negative regulation of the

myeloid dendritic cell lineage. *J Leukoc Biol* 1999; **66**: 209-216 [PMID: 10449156]
2 **Chaussabel D**, Banchereau J. Dendritic cells, therapeutic vectors of immunity and tolerance. *Am J Transplant* 2005; **5**: 205-206 [PMID: 15643979 DOI: 10.1111/j.1600-6143.2004.00784.x]
3 **Banchereau J**, Palucka AK. Dendritic cells as therapeutic vaccines against cancer. *Nat Rev Immunol* 2005; **5**: 296-306 [PMID: 15803149 DOI: 10.1038/nri1592]
4 **Jonuleit H**, Schmitt E, Schuler G, Knop J, Enk AH. Induction of interleukin 10-producing, nonproliferating CD4(+) T cells with regulatory properties by repetitive stimulation with allogeneic immature human dendritic cells. *J Exp Med* 2000; **192**: 1213-1222 [PMID: 11067871 DOI: 10.1084/jem.192.9.1213]
5 **van Duivenvoorde LM**, van Mierlo GJ, Boonman ZF, Toes RE. Dendritic cells: vehicles for tolerance induction and prevention of autoimmune diseases. *Immunobiology* 2006; **211**: 627-632 [PMID: 16920501 DOI: 10.1016/j.imbio.2006.05.014]
6 **O'Connell RM**, Taganov KD, Boldin MP, Cheng G, Baltimore D. MicroRNA-155 is induced during the macrophage inflammatory response. *Proc Natl Acad Sci USA* 2007; **104**: 1604-1609 [PMID: 17242365 DOI: 10.1073/pnas.0610731104]
7 **Thai TH**, Calado DP, Casola S, Ansel KM, Xiao C, Xue Y, Murphy A, Frendewey D, Valenzuela D, Kutok JL, Schmidt-Supprian M, Rajewsky N, Yancopoulos G, Rao A, Rajewsky K. Regulation of the germinal center response by microRNA-155. *Science* 2007; **316**: 604-608 [PMID: 17463289 DOI: 10.1126/science.1141229]
8 **Rodriguez A**, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR, van Dongen S, Grocock RJ, Das PP, Miska EA, Vetrie D, Okkenhaug K, Enright AJ, Dougan G, Turner M, Bradley A. Requirement of bic/microRNA-155 for normal immune function. *Science* 2007; **316**: 608-611 [PMID: 17463290 DOI: 10.1126/science.1139253]
9 **Vigorito E**, Perks KL, Abreu-Goodger C, Bunting S, Xiang Z, Kohlhaas S, Das PP, Miska EA, Rodriguez A, Bradley A, Smith KG, Rada C, Enright AJ, Toellner KM, Maclennan IC, Turner M. microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. *Immunity* 2007; **27**: 847-859 [PMID: 18055230 DOI: 10.1016/j.immuni.2007.10.009]
10 **Wu L**, Liu YJ. Development of dendritic-cell lineages. *Immunity* 2007; **26**: 741-750 [PMID: 17582346 DOI: 10.1016/j.immuni.2007.06.006]
11 **Carotta S**, Dakic A, D'Amico A, Pang SH, Greig KT, Nutt SL, Wu L. The transcription factor PU.1 controls dendritic cell development and Flt3 cytokine receptor expression in a dose-dependent manner. *Immunity* 2010; **32**: 628-641 [PMID: 20510871 DOI: 10.1016/j.immuni.2010.05.005]
12 **McKercher SR**, Torbett BE, Anderson KL, Henkel GW, Vestal DJ, Baribault H, Klemsz M, Feeney AJ, Wu GE, Paige CJ, Maki RA. Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. *EMBO J* 1996; **15**: 5647-5658 [PMID: 8896458]
13 **Lutz MB**, Suri RM, Niimi M, Ogilvie AL, Kukutsch NA, Rössner S, Schuler G, Austyn JM. Immature dendritic cells generated with low doses of GM-CSF in the absence of IL-4 are maturation resistant and prolong allograft survival in vivo. *Eur J Immunol* 2000; **30**: 1813-1822 [PMID: 10940870]
14 **Yang XJ**, Meng S, Zhu CF, Jiang H, Wu WX. Semi-mature MyD88-silenced bone marrow dendritic cells prolong the allograft survival in a rat model of intestinal transplantation. *Chin Med J* (Engl) 2011; **124**: 268-272 [PMID: 21362379]
15 **Hirose S**, Nishizumi H, Sakano H. Pub, a novel PU.1 binding protein, regulates the transcriptional activity of PU.1. *Biochem Biophys Res Commun* 2003; **311**: 351-360 [PMID: 14592421 DOI: 10.1016/j.bbrc.2003.09.212]
16 **Zhang X**, Li J, Li N, Shi X, Li Y. Safety of enteral rehabilitative therapy in rat small bowel transplantation. *Chin Med J* (Engl) 2003; **116**: 703-707 [PMID: 12875685]
17 **McCurry KR**, Colvin BL, Zahorchak AF, Thomson AW. Regulatory dendritic cell therapy in organ transplantation. *Transpl Int* 2006; **19**: 525-538 [PMID: 16764631 DOI: 10.1111/j.1432-2277.2006.00306.x]
18 **Casiraghi F**, Aiello S, Remuzzi G. Transplant tolerance: progress and challenges. *J Nephrol* 2010; **23**: 263-270 [PMID: 20349420]
19 **Vosters O**, Nève J, De Wit D, Willems F, Goldman M, Verhasselt V. Dendritic cells exposed to nacystelyn are refractory to maturation and promote the emergence of alloreactive regulatory t cells. *Transplantation* 2003; **75**: 383-389 [PMID: 12589163 DOI: 10.1097/01.TP.0000043924.09647.61]
20 **Mahnke K**, Schmitt E, Bonifaz L, Enk AH, Jonuleit H. Immature, but not inactive: the tolerogenic function of immature dendritic cells. *Immunol Cell Biol* 2002; **80**: 477-483 [PMID: 12225384 DOI: 10.1046/j.1440-1711.2002.01115.x]
21 **Lutz MB**, Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? *Trends Immunol* 2002; **23**: 445-449 [PMID: 12200066 DOI: 10.1016/S1471-4906(02)02281-0]
22 **Akdis CA**, Blaser K. Mechanisms of interleukin-10-mediated immune suppression. *Immunology* 2001; **103**: 131-136 [PMID: 11412299 DOI: 10.1046/j.1365-2567.2001.01235.x]
23 **Miller G**, Pillarisetty VG, Shah AB, Lahrs S, DeMatteo RP. Murine Flt3 ligand expands distinct dendritic cells with both tolerogenic and immunogenic properties. *J Immunol* 2003; **170**: 3554-3564 [PMID: 12646617]
24 **Yamazaki S**, Inaba K, Tarbell KV, Steinman RM. Dendritic cells expand antigen-specific Foxp3+ CD25+ CD4+ regulatory T cells including suppressors of alloreactivity. *Immunol Rev* 2006; **212**: 314-329 [PMID: 16903923 DOI: 10.1111/j.0105-2896.2006.00422.x]
25 **Rössner S**, Voigtländer C, Wiethe C, Hänig J, Seifarth C, Lutz MB. Myeloid dendritic cell precursors generated from bone marrow suppress T cell responses via cell contact and nitric oxide production in vitro. *Eur J Immunol* 2005; **35**: 3533-3544 [PMID: 16331707 DOI: 10.1002/eji.200526172]

P- Reviewers: Detry Olivier, Pei ZH **S- Editor:** Ma YJ
L- Editor: Wang TQ **E- Editor:** Wu HL

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v19.i43.7772

World J Gastroenterol 2013 November 21; 19(43): 7772-7777
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Biliary casts after liver transplantation: Morphology and biochemical analysis

Yu-Long Yang, Cheng Zhang, Mei-Ju Lin, Li-Jun Shi, Hong-Wei Zhang, Jing-Yi Li, Qiang Yu

Yu-Long Yang, Cheng Zhang, Mei-Ju Lin, Li-Jun Shi, Hong-Wei Zhang, Jing-Yi Li, Qiang Yu, Department of Minimally Invasive Biliary Surgery, Affiliated Zhongshan Hospital of Dalian University, Dalian 116001, Liaoning Province, China

Author contributions: Yang YL conceived and designed the study; Yang YL, Zhang C, Lin MJ, Shi LJ, Zhang HW, Li JY and Yu Q performed the examinations; Yang YL, Lin MJ, Shi LJ, Zhang HW and Li JY performed the surgery; and Zhang C wrote the manuscript.

Correspondence to: Yu-Long Yang, PhD, Department of Minimally Invasive Biliary Surgery, Affiliated Zhongshan Hospital of Dalian University, Jiefang Street, No.6, Zhongshan District, Dalian 116001, Liaoning Province, China. yyl516@126.com
Telephone: +86-411-62893656 Fax: +86-411-62893555
Received: April 24, 2013 Revised: September 24, 2013
Accepted: September 29, 2013
Published online: November 21, 2013

Abstract

AIM: To investigate the pathogenesis of biliary casts after liver transplantation relative to their morphology and biochemical markers.

METHODS: The microstructure of biliary casts was assessed using scanning electron microscopy and Hematoxylin and eosin staining assessed their histology. The expression levels of CD3, CD5, CD34, CD68 and CD79a in these biliary casts were evaluated immunohistochemically.

RESULTS: Biliary casts differed widely in their microstructure, with some containing blood vessels positive for CD34 and collagen fibers with positive Masson staining. Large numbers of neutrophils and other inflammatory cells were present, but only on the edge of the biliary casts; although the boundaries were clear without crossover. None of the biliary casts contained T-lymphocytes, B-lymphocytes, macrophages and other inflammatory cells.

CONCLUSION: The microcostructure of biliary casts differed. Bacteria and acute rejection are not clearly related to their formation.

Key words: Biliary cast; Biliary cast syndrome; Liver transplantation; Blood vessels; Acute rejection

Core tip: This experimental study employed scanning electron microscopy, Hematoxylin and eosin staining and immunohistochemistry to investigate biliary casts following liver transplantation. The results indicated that blood vessels and collagen fibers are present in biliary casts; however, bacteria and acute rejection are not clearly related to their formation, as evidenced by blood vessels positive for CD34 and collagen fibers with positive Masson staining, and no T-lymphocytes, B-lymphocytes, macrophages and other inflammatory cells. Thus, although bile duct injury after liver transplantation is significantly associated with biliary cast formation, their role in acute rejection is unclear.

Yang YL, Zhang C, Lin MJ, Shi LJ, Zhang HW, Li JY, Yu Q. Biliary casts after liver transplantation: morphology and biochemical analysis. *World J Gastroenterol* 2013; 19(43): 7772-7777 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7772.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7772

INTRODUCTION

Despite advances in the management of patients who have undergone cadaveric liver transplantation, 6%-34% patients experience biliary complications[1]. Biliary cast syndrome (BCS), first described in 1975[2], occurs less frequently than biliary sludge and stones, with an incidence of 2.5% after orthotopic liver transplantation[3]. Multiple intrahepatic biliary strictures, ductal dilatation,

intrahepatic abscesses, and biliary anastomotic leakage characterize BCS. The clinical symptoms of BCS usually include high fever, jaundice and cholestatic liver enzyme elevation, similar to the symptoms observed in some patients with intrahepatic bile duct stones. Surgical management is the treatment of choice, and endoscopic techniques have been successful and safe in the removal of biliary casts[4-6]. Morphologically, biliary casts are a similar shape to bile ducts, appearing as a hardened, dark material in the biliary ductal system. Biliary casts can prevent bile drainage, resulting in biliary obstruction and inducing biliary tract infection. Biliary casts can ultimately cause substantial injury to the liver, with some transplant recipients requiring retransplantation. Although the associations between biliary casts and clinical treatment have been assessed recently, less is known about the associations between biliary casts and biochemical markers. We therefore investigated the pathogenesis of biliary casts after liver transplantation relative to their morphology and biochemical markers.

MATERIALS AND METHODS

Isolation of biliary casts

We evaluated 15 patients with a history of orthotopic liver transplantation, who were treated in our department for jaundice, recurrent cholangitis and high fever. There were 10 males and 5 females, with a mean age of 52.1 years (range, 34-78 years). Of these patients, five underwent deceased donor liver transplantation for hepatitis B-induced cirrhosis and primary liver cancer, one for primary hepatocellular carcinoma and nine for cirrhosis during the decompensated period. Choledochoscopy and duodenoscopy have been used frequently to assess patients with biliary complications after liver transplantation[7,8]. Patients with T-tube fistulae can be evaluated by insertion of a cholangioscope directly into the common hepatic duct, whereas patients without T-tube fistulae are evaluated preferably by percutaneous transhepatic cholangioscopy or endoscopic retrograde cholangiopancreatography[9]. The distal aspect of the cast was secured using a basket, allowing each cast to be successfully removed as a single piece. All the casts were stored in liquid nitrogen.

Scanning electron microscopy

Following their isolation, biliary casts that were kept at room temperature were rinsed in sterile normal saline solution, fixed with 10% neutral formalin for 12 h at 4 ℃, rinsed in 0.1 mol/L phosphate buffer (pH 7.0) and dehydrated through a graded series of ethanol (10 min each at 10%, 30%, 50%, 70% and 90%, and 15 min each three times at 100%). After critical point drying at 30 ℃ with CO_2 for 6 h, the samples were mounted, coated with 1-μm gold particles and evaluated using a Hitachi S 4800 field emission scanning electron microscope at 2 kV.

Histological and immunohistochemical examination

Biliary casts stored in liquid nitrogen were rinsed in sterile normal saline solution, fixed with 10% neutral formalin for 12 h at 4 ℃, embedded in paraffin, cross-sectioned into 10 mm slices and placed onto glass slides. Some of these histological sections were stained with hematoxylin and eosin (HE) and Masson trichrome, according to standard procedures. The remaining histological sections were deparaffinized, rehydrated, incubated in 3% hydrogen peroxide/absolute methanol for 5 min to block endogenous peroxidase activity and rinsed in distilled water. Nonspecific binding of antibodies was blocked by incubation with 5% normal goat serum for 10 min at room temperature. After washing, the sections were incubated with primary rabbit antibodies against human CD3, CD5, CD34, CD68 and CD79a, overnight at 4 ℃. The sections were subsequently incubated with biotinylated secondary antibody for 30 min at 37 ℃, with streptavidin biotin complex reagent for 30 min at 37 ℃, and with DAB Plus reagent for 10 min, with the sections repeatedly washed with PBS, pH 7.4, between incubations. The sections were counterstained with hematoxylin, mounted and examined by optical microscopy. All antibodies and reagents for immunohistochemistry were purchased from the Beijing Zhongshan Golden Bridge Biotechnology Company, Beijing, China.

RESULTS

Biliary casts have a variety of morphological structures

Morphologically, biliary casts have a cordlike, columnar, dendritic shape within the biliary ductal system (Figure 1A). Scanning electron microscopy, however, showed that biliary casts were present in a variety of forms: irregular sheets composed of imbricated accumulations (Figure 1B); honeycombs with porous structures and adherent crystalline substances (Figure 1C); and filamentous structures (Figure 1D).

Biliary casts contain blood vessels and collagen fibers

HE staining revealed large numbers of lacunae containing bilirubin, tubiform (Figure 2A) and filamentous structures (Figure 2B). To determine the composition of the tubiform and filamentous structures, we incubated these sections with antibodies to cell markers and with Masson stain. We found that the tubiform structures were positive for CD34 (Figure 2C), whereas the filamentous structures were positive for Masson stain (Figure 2D). These findings indicated that the tubiform structures were blood vessels and the filamentous structures were collagen fibers.

Formation of biliary casts is not related to inflammatory response

HE staining showed large numbers of neutrophils and other inflammatory cells on the edge of the biliary casts; however, the boundaries were clear without crossover, and no inflammatory cells were present within the biliary casts (Figure 2E). Scanning electron microscopy showed no evidence of bacteria or bacterial debris on the surface of the biliary casts.

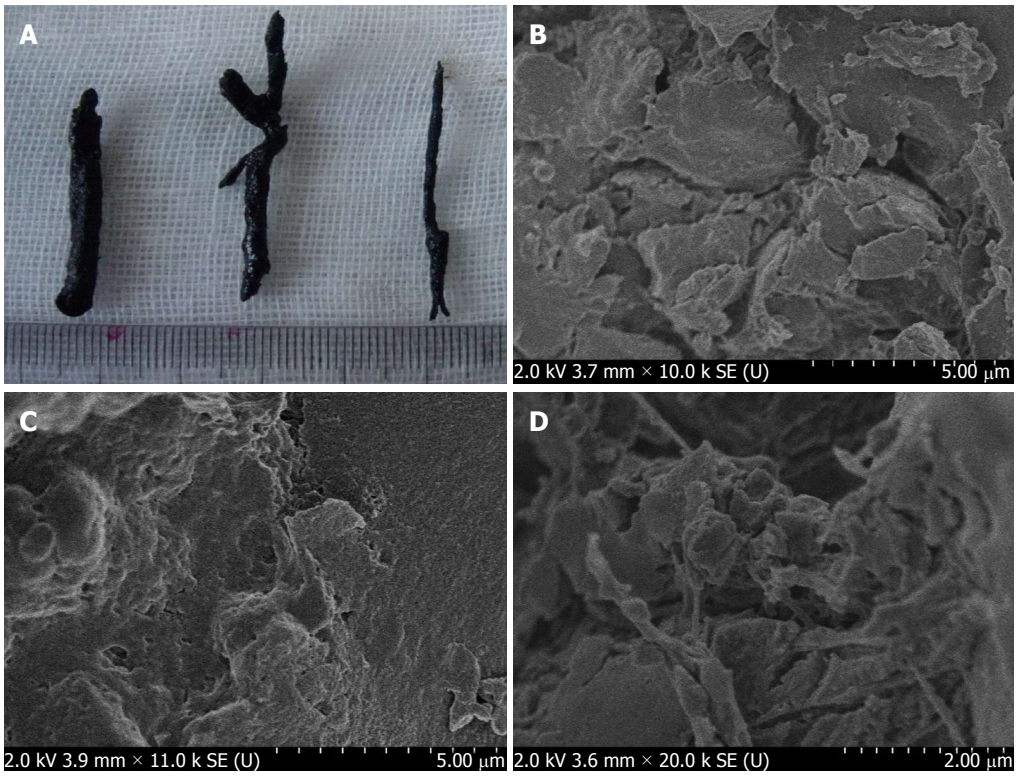


Figure 1 Morphology of biliary casts. A: Cordlike, columnar and dendritic shapes of biliary casts within the biliary ductal system; B: A biliary cast in the shape of an irregular sheet composed of imbricated accumulation (× 10000); C: A biliary cast in the shape of a honeycombs with porous structures and adherent crystalline substances (× 10000); D: A biliary cast in the shape of filamentous structures (× 10000).

Immune rejection is not significantly related to biliary cast formation

Acute rejection generally occurs 1 to 3 wk after liver transplantation. To determine the relationship between immune rejection reactions and biliary cast formation, we incubated the biliary cast samples with antibodies to CD3, CD5, CD68, and CD79a. None of the biliary casts was positive for any of these markers, indicating that these biliary casts did not contain T-lymphocytes, B-lymphocytes and macrophages.

DISCUSSION

Physically and morphologically, biliary casts appear as dark, hardened material in the shape of bile ducts within the biliary ductal system, but differ from bile duct stones. Scanning electron microscopy showed that biliary casts appear in a variety of forms, including irregular sheets composed of imbricated accumulations; honeycombs with a porous structure and adherent crystalline substances; and filamentous structures. Although bile duct stones and biliary casts have a similar microstructure[10], their mechanism of formation differs significantly. Biliary casts that form after liver transplantation are not caused by a single pathogenic factor, but may be associated with late functional rehabilitation, biliary strictures and obstruction, acute rejection, recurrent cholangitis, cold and warm ischemia times, hepatic ischemia and reperfusion injury[3,11-13].

Bilirubin has been reported to be the primary component of biliary casts (approximately 10%-50%), followed by bile acid synthesis products and cholesterol, with protein comprising only 5%-10%[14]. In comparison, we observed large amounts of bilirubin, as well as blood vessels and collagen fibers, consistent with our earlier findings. Choledochoscopy showed a large number of flocs in bile duct cellulose 5 mo after transplantation, with histopathological examination showing that these flocs were composed of cellulose, bile duct epithelial cells and necrotic inflammatory cells. These elements then become structureless, with biliary casts observed in the bile ducts 9 mo after transplantation[15]. The presence of blood vessels and collagen fibers in the biliary casts was related to injury to the bile duct mucosa. The extent of bile duct injury during orthotopic liver transplantation differs, with cold preservation/reperfusion injury being the most important initiator of bile duct tree injuries and vessel plexus damage. Bile duct injury may, therefore, be associated with microcirculatory disturbances surrounding the bile ducts[16]; however, the specific mechanisms underlying bile duct injury require further investigation.

Acute rejection after liver transplantation generally occurs 1 to 3 wk postoperatively. Typical clinical symptoms include unexplained fever, loss of appetite, poor spirit, liver pain, progressively deepening jaundice, and elevated bilirubin and transaminase. The diagnosis mainly depends on liver puncture biopsy and pathology. Biliary casts and acute rejection after transplantation have

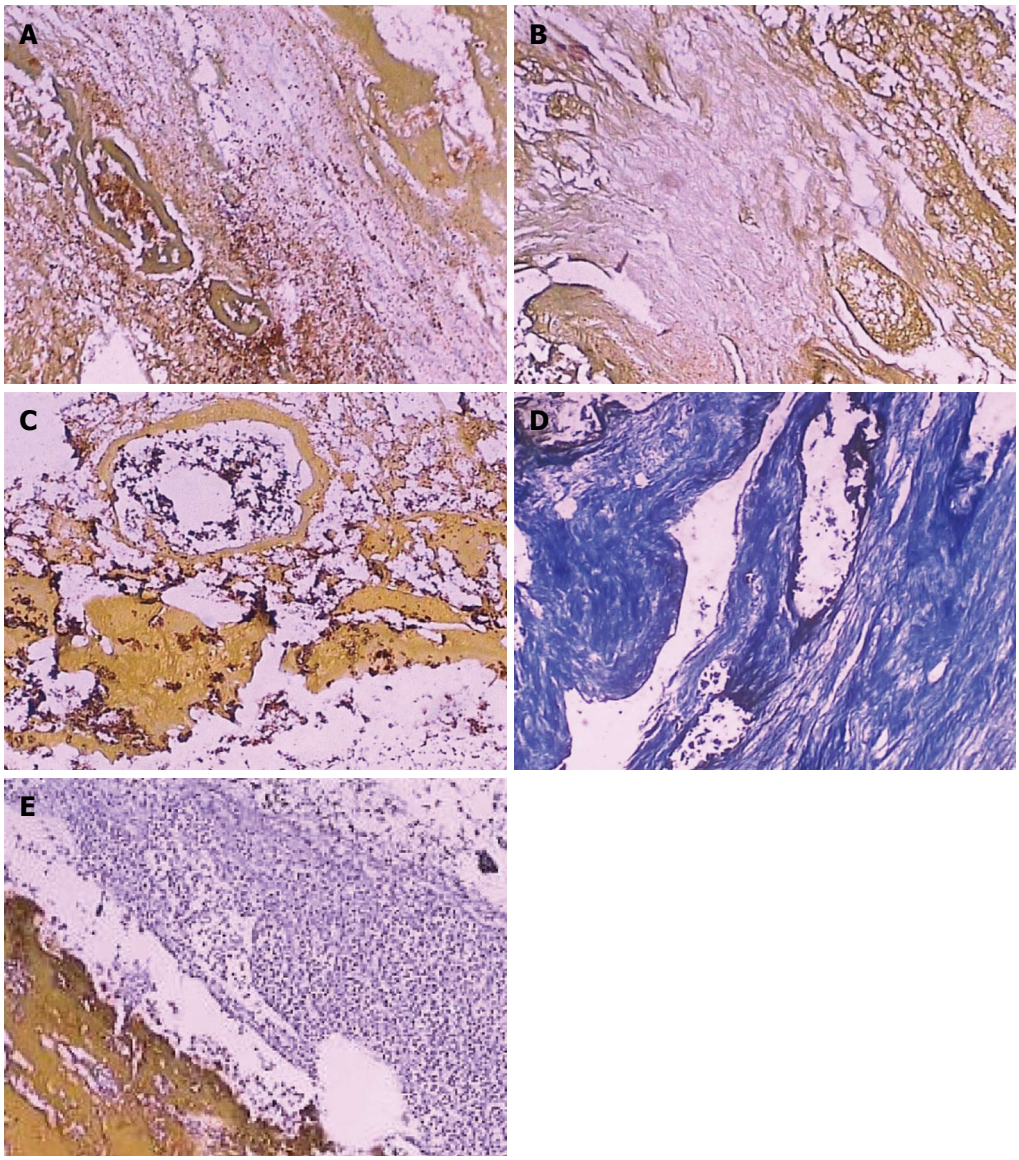


Figure 2 Histological and immunohistochemical examination of biliary casts. A: Histological examination of a biliary cast, showing tubiform structures (HE staining × 100); B: Histological examination of a biliary cast, showing filamentous structures (HE staining × 100); C: A biliary cast with tubiform structures positive for CD34 (brown color, × 100); D: A biliary cast with filamentous structures positive for collagen fibers (Masson staining × 100); E: A biliary cast showing peripheral positivity for neutrophils and other inflammatory cells (HE staining, × 100).

a similar time of onset and similar clinical symptoms. However, biliary casts generally form at least 1 mo after transplantation[17]. Liver recipients with high serum concentrations of soluble major histocompatibility complex class I related chain A (sMICA) tend to develop BCS more easily than recipients with normal post-transplant sMICA concentrations[18]. We hypothesized that the formation of biliary casts was related to acute rejection and that T lymphocytes, B lymphocytes and macrophages would be present in biliary casts. However, we found that these cells were absent from biliary casts arising after liver transplantation, similar to the findings in patients who underwent non-liver transplantation[19,20]. Therefore, our findings suggest that acute rejection after liver transplantation was not significantly associated with biliary cast formation.

Electron microscopic examination of cholesterol calculi showed the presence of bacteria in the core and periphery of cholesterol stones, suggesting that bacteria may be involved in initiating the formation of cholesterol stones[21,22]. Patients with biliary casts usually have recurrent episodes of cholangitis. *Escherichia coli*, which has glucuronidase activity and can grow in cultures of biliary casts, can degrade conjugated bile acids and conjugated bilirubin, yielding free bile acids and free bilirubin, respectively. Free bile acids and free bilirubin are relatively insoluble and are not present in the bile of patients. Damage to the bile duct mucosa can result in their precipitation into biliary casts, suggesting that a number of factors, including infection, supersaturation with cholesterol and mucosal damage, may be involved in bile cast formation after liver transplantation[2]. To assess the relationship between bacteria and biliary casts, we evaluated biliary casts using scanning electron mi-

croscopy. However, neither bacteria nor bacterial debris was observed in the interior or surface of biliary casts. Large numbers of neutrophils were observed on the periphery of biliary casts, but the boundaries were clear and there were no neutrophils or similar cells within the mold. The multiplication of bacteria in an environment of poor bile drainage and cholestasis caused by biliary casts may therefore induce recurrent fever, obstructive jaundice and other complications. Biliary tract infections may be secondary pathological changes following biliary cast formation, rather than being the direct cause of mold formation. Therefore, when treating patients who experience complications after liver transplantation, anti-infectious agents may only alleviate the symptoms. The removal of the biliary casts may therefore be primary.

COMMENTS

Background

Biliary casts are infrequent complications after liver transplantation, resulting in various clinical symptoms. Although the associations between biliary casts and clinical treatment have been assessed recently, less is known about the associations between biliary casts and biochemical markers.

Research frontiers

The current pathogenesis study of biliary casts after liver transplantation mostly concentrated on clinical aspects. Biliary casts were not caused by a single pathogenic factor, but may be associated with late functional rehabilitation, biliary strictures and obstruction, acute rejection, recurrent cholangitis, cold and warm ischemia times, hepatic ischemia and reperfusion injury.

Innovations and breakthroughs

The results indicated that blood vessels and collagen fibers are present in biliary casts; however, bacteria and acute rejection are not clearly related to their formation, as evidenced by blood vessels positive for CD34 and collagen fibers with positive Masson staining, and the absence of T-lymphocytes, B-lymphocytes, macrophages and other inflammatory cells.

Applications

These findings indicate that bile duct injury is clearly associated with biliary cast formation after liver transplantation; however, bacteria and acute rejection were not significantly related to their formation.

Terminology

Biliary cast syndrome, first described in 1975, occurs less frequently than biliary sludge and stones, with an incidence of 2.5% after orthotopic liver transplantation. Orthotopic liver transplantation refers to a procedure in which a failed liver is removed from the patient's body and a healthy donor liver is transplanted into the same location. Biliary casts are infrequent complications after liver transplantation, resulting in various clinical symptoms.

Peer review

The authors analyzed the pathogenesis of biliary casts after liver transplantation relative to their morphology and biochemical markers. These findings indicate that bile duct injury was clearly associated with biliary cast formation after liver transplantation, but that bacteria and acute rejection are not clearly related to their process of bile duct injury. Therefore, it is an interesting study. The analytical approaches are described in detail, and the results are impressive.

REFERENCES

1 **Bak SH**, Choi HS, Yang SY, Jun DW, Han SH, Lee HL, Lee OY, Yoon BC, Hahm JS, Lee MH, Lee DH, Kee CS. [Obstructive jaundice due to biliary cast syndrome followed by orthotopic liver transplantation]. *Korean J Gastroenterol* 2006; **48**: 119-123 [PMID: 16929156]

2 **Waldram R**, Williams R, Calne RY. Bile composition and bile cast formation after transplantation of the liver in man. *Transplantation* 1975; **19**: 382-387 [PMID: 168674]

3 **Gor NV**, Levy RM, Ahn J, Kogan D, Dodson SF, Cohen SM. Biliary cast syndrome following liver transplantation: Predictive factors and clinical outcomes. *Liver Transpl* 2008; **14**: 1466-1472 [PMID: 18825683 DOI: 10.1002/lt.21492]

4 **Navaneethan U**, Venkatesh PG, Al Mohajer M, Gelrud A. Successful diagnosis and management of biliary cast syndrome in a liver transplant patient using single operator cholangioscopy. *JOP* 2011; **12**: 461-463 [PMID: 21904071]

5 **Saleem A**, Baron TH. Successful endoscopic treatment of biliary cast syndrome in an orthotopic liver transplant patient with a Roux-en-Y anastomosis via balloon enteroscopy. *Liver Transpl* 2010; **16**: 527-529 [PMID: 20213831 DOI: 10.1002/lt.22007]

6 **Srinivasaiah N**, Reddy MS, Balupuri S, Talbot D, Jaques B, Manas D. Biliary cast syndrome: literature review and a single centre experience in liver transplant recipients. *Hepatobiliary Pancreat Dis Int* 2008; **7**: 300-303 [PMID: 18522886]

7 **Barritt AS**, Miller CB, Hayashi PH, Dellon ES. Effect of ERCP utilization and biliary complications on post-liver-transplantation mortality and graft survival. *Dig Dis Sci* 2010; **55**: 3602-3609 [PMID: 20411423 DOI: 10.1007/s10620-010-1213-6]

8 **Yang Yl**, Fu QL, Tan WX, Liu XB, Feng QS, Zhang BS. Diagnosis and treatment of 6 patients with biliary complications following orthotopic liver transplantation by choledochofiberscopy. *Zhonghua Qiguan Yizhi Zazhi* 2005; **26**: 561-563 [DOI: 10.3760/cma.j.issn.0254-1785.2005.09.014]

9 **Yu Q**, Yang YL, Lin MJ, Zhang HW, Shi LJ, Li JY, Zhang C. The therapeutic value of percutaneous transhepatic cholangioscopy for biliary cast after liver transplantation. *Zhonghua Xiaohua Neijing Zazhi* 2011; **28**: 146-149 [DOI: 10.3760/cma.j.issn.1007-5232.2011.03.012]

10 **Yu Q**, Yang YL, Lin MJ, Zhang HW, Shi LJ. A Comparative Study of liver transplantation biliary cast and morphology of bile duct stones. *Zhonghua Waike Zazhi* 2011; **49**: 650-651 [DOI: 10.3760/cma.j.issn.0529-5815.2011.07.018]

11 **Starzl TE**, Putnam CW, Hansbrough JF, Porter KA, Reid HA. Biliary complications after liver transplantation: with special reference to the biliary cast syndrome and techniques of secondary duct repair. *Surgery* 1977; **81**: 212-221 [PMID: 319551]

12 **Sawyer RG**, Punch JD. Incidence and management of biliary complications after 291 liver transplants following the introduction of transcystic stenting. *Transplantation* 1998; **66**: 1201-1207 [PMID: 9825818]

13 **Sheng R**, Ramirez CB, Zajko AB, Campbell WL. Biliary stones and sludge in liver transplant patients: a 13-year experience. *Radiology* 1996; **198**: 243-247 [PMID: 8539387]

14 **Shah JN**, Haigh WG, Lee SP, Lucey MR, Brensinger CM, Kochman ML, Long WB, Olthoff K, Shaked A, Ginsberg GG. Biliary casts after orthotopic liver transplantation: clinical factors, treatment, biochemical analysis. *Am J Gastroenterol* 2003; **98**: 1861-1867 [PMID: 12907345 DOI: 10.1111/j.1572-0241.2003.07617.x]

15 **Yang YL**, Fu WL, Liu XB, Tan WX, Zhang BS, Feng QS. Diagnosis and treatment of biliary stone by choledochofibroscope after orthotopic liver transplantation. *Zhonghua Gandan Waike Zazhi* 2005; **11**: 528-529 [DOI: 10.3760/cma.j.issn.1007-8118.2005.08.007]

16 **Tan WX**, Yang YL, Wang XG, Fu WL. Pathological changes of bile duct injury after orthotopic liver transplantation. *Zhongguo Zuzhi Gongcheng Yanjiu yu Linchuang Kangfu* 2008; **12**: 10583-10587

17 **Yang YL**, Liu ZW, Lin MJ, Zhang HW, Shi LJ. Analysis of liver function test and screening of early warning indicators for biliary cast group after orthotopic liver transplantation. *Zhongguo Zuzhi Gongcheng Yanjiu yu Linchuang Kangfu* 2011; **15**: 5707-5712 [DOI: 10.3969/j.issn.1673-8225.2011.31.002]

18 **Zou Y**, Yang X, Jiang X, Wang H, Hao Q, Liu Y, Yu P. High levels of soluble Major Histocompatibility Complex class I related chain A (MICA) are associated with biliary cast syndrome after liver transplantation. *Transpl Immunol* 2009; **21**: 210-214 [PMID: 19539762 DOI: 10.1016/j.trim.2009.06.003]

19 **Zimmer V**, Raedle J, Treiber G, Lammert F. Biliary cast syndrome in sclerosing cholangitis. *Dig Liver Dis* 2011; **43**: e4 [PMID: 20207597 DOI: 10.1016/j.dld.2010.01.012]
20 **Gleeson FC**, Czaja AJ, Baron TH. Successful endoscopic management of biliary cast syndrome in nonliver transplant patients. *J Clin Gastroenterol* 2008; **42**: 752-755 [PMID: 18496388 DOI: 10.1097/01.mcg.0000225626.76544.67]
21 **Yang YL**, Tan WX, Liu XB, Li XT. Observation of Bacteria in Cholesterol Stones by Electronic Microscope. *Zhongguo Puwai Jichu yu Linchuang Zazhi* 2004; **5**: 447-449 [DOI: 10.3969/j.issn.1007-9424.2004.05.022]
22 **Wu SD**, Yu H, Su Y, Zhang ZH, Fan Y, Sun SL, Kong J, Tian Y, Tian Z, Wei Y, Zhao XS, Jin JJ, Zhang Q. Study on etiology of primary bile duct pigment stones. *Zhonghua Gandan Waike Zazhi* 2007; **13**: 717-720 [DOI: 10.3760/cma.j.issn.1007-8118.2007.08.006]

P- Reviewers: Nundy S, Sugawara Y, Tolba RH
S- Editor: Song XX **L- Editor:** Stewart G **E- Editor:** Liu XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v19.i43.7778

World J Gastroenterol 2013 November 21; 19(43): 7778-7787
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Amelioration of carbon tetrachloride-induced cirrhosis and portal hypertension in rat using adenoviral gene transfer of Akt

Gang Deng, Xiang-Jun Huang, Hong-Wu Luo, Fei-Zhou Huang, Xun-Yang Liu, Yong-Heng Wang

Gang Deng, Xiang-Jun Huang, Hong-Wu Luo, Fei-Zhou Huang, Xun-Yang Liu, Yong-Heng Wang, Department of Hepatobiliary and Pancreatic Surgery, the Third Xiangya Hospital of Central South University, Changsha 410013, Hunan Province, China

Author contributions: Huang FZ, Liu XY and Wang YH participated in research design and other authors collectively contributed to the performance of laboratory measurements; Deng G, Huang XJ and Huang FZ were involved in data collection and analysis; and Deng G, Huang XJ and Luo HW wrote the manuscript.

Correspondence to: Hong-Wu Luo, MD, Department of Hepatobiliary and Pancreatic Surgery, the Third Xiangya Hospital of Central South University, Changsha 410013, Hunan Province, China. luohongwuzny@sina.com

Telephone: +86-731-88618230 Fax: +86-731-88618030
Received: June 30, 2013 Revised: August 28, 2013
Accepted: September 15, 2013
Published online: November 21, 2013

Abstract

AIM: To investigate whether a virus constitutively expressing active Akt is useful to prevent cirrhosis induced by carbon tetrachloride (CCl_4).

METHODS: Using cre-loxp technique, we created an Ad-myr-HA-Akt virus, in which Akt is labeled by a HA tag and its expression is driven by myr promoter. Further, through measuring enzyme levels and histological structure, we determined the efficacy of this Ad-myr-HA-Akt virus in inhibiting the development of cirrhosis induced by CCl_4 in rats. Lastly, using western blotting, we examined the expression levels and/or phosphorylation status of Akt, apoptotic mediators, endothelial nitric oxide synthase (eNOS), and markers for hepatic stellate cells activation to understand the underlying mechanisms of protective role of this virus.

RESULTS: The Ad-myr-HA-Akt virus was confirmed using polymerase chain reaction amplification of inserted *Akt* gene and sequencing for full length of inserted fragment, which was consistent with the sequence reported in the GenBank. The concentrations of Ad-myr-HA-Akt and adenoviral enhanced green fluorescent protein (Ad-EGFP) virus used in the current study were 5.5×10^{11} vp/mL. The portal vein diameter, peak velocity of blood flow, portal blood flow and congestion index were significantly increased in untreated, saline and Ad-EGFP cirrhosis groups when compared to normal control after the virus was introduced to animal through tail veil injection. In contrast, these parameters in the Akt cirrhosis group were comparable to normal control group. Compared to the normal control, the liver function (Alanine aminotransferase, Aspartate aminotransferase and Albumin) was significantly impaired in the untreated, saline and Ad-EGFP cirrhosis groups. The Akt cirrhosis group showed significant improvement of liver function when compared to the untreated, saline and Ad-EGFP cirrhosis groups. The Hyp level and portal vein pressure in Akt cirrhosis groups were also significantly lower than other cirrhosis groups. The results of HE and Van Gieson staining indicated that Akt group has better preservation of histological structure and less fibrosis than other cirrhosis groups. The percentage of apoptotic cell was greatly less in Akt cirrhosis group than in other cirrhosis groups. Akt group showed positive HA tag and an increased level of phosphorylated Akt as well as decreased levels of Fas. In contrast, Caspase-3 and Caspase-9 levels in Akt group were significantly lower than other cirrhosis groups. Noticeable decrease of DR5 and α-SMA and increase of phosphorylated eNOS were observed in the Akt group when compared to other cirrhosis groups. The NO level in liver was significantly higher in Akt group than other cirrhosis groups, which was consistent with the level of phosphorylated eNOS in these groups.

CONCLUSION: This study suggest that Ad-myr-HA-Akt virus is a useful tool to prevent CCl_4-induced cirrhosis in rat model and Akt pathway may be a therapeutic target

for human cirrhosis.

Key words: Adenovirus; Akt; Gene transfer; Apoptosis; Cirrhosis; Carbon tetrachloride; Rat

Core tip: In the present study, we have demonstrated for the first time that Ad-myr-HA-Akt virus was a useful tool to prevent carbon tetrachloride-induced cirrhosis in rat model. Our data obtained at different levels, from function to histological changes, apoptosis rate of hepatocytes, activation of hepatic stellate cells, deposition of collagen, portal vein pressure and NO level, which were all consistently and collectively supported the hypothesis that introduction of Ad-myr-HA-Akt virus inhibits the development of cirrhosis.

Deng G, Huang XJ, Luo HW, Huang FZ, Liu XY, Wang YH. Amelioration of carbon tetrachloride-induced cirrhosis and portal hypertension in rat using adenoviral gene transfer of Akt. *World J Gastroenterol* 2013; 19(43): 7778-7787 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7778.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7778

INTRODUCTION

It has been found that massive apoptosis of hepatocytes resulted in activation of hepatic stellate cells (HSC), which produce collagen and ultimately lead to the fibrosis and cirrhosis[1-5]. Accordingly, inhibiting hepatic apoptosis is considered as an important strategy to prevent cirrhosis[6-10]. Previous research showed that Akt plays a crucial role in preventing Fas signaling-mediated hepatic apoptosis, and that over-expression of Akt was capable of preventing hepatic apoptosis[11,12]. Therefore, we intended to establish a recombinant vector carrying *Akt* gene to inhibit cirrhosis in a rat model.

Adenovirus can effectively express genes of interest. Accordingly it has been widely used as vector for gene therapy[13-18]. Recently, Cre-loxp system has been used to create replication-defective virus and successfully used in creating therapeutic virus with recombinant gene[19-22]. In the present study, using cre-loxp technique, we created an Ad-myr-HA-Akt virus, in which Akt is labeled by a Hemagglutinin (HA) tag and its expression is driven by myr promoter. We further determined that this Ad-myr-HA-Akt virus was capable of inhibiting the development of cirrhosis induced by carbon tetrachloride (CCl_4). Lastly, we examined the expression level and/or phosphorylation status of Akt, apoptotic mediators, endothelial nitric oxide synthase (eNOS), and markers for HSC activation. Our results indicated that introduction of Ad-myr-HA-Akt virus was able to prevent hepatocyte apoptosis and subsequent cirrhosis in a rat CCl_4-induced cirrhosis model.

MATERIALS AND METHODS

Preparation of recombinant adenoviral vector carrying Akt

According to the method that has been published[23], a replication-defective adenovirus vector was constructed in HEK293 cell with co-transfection of pCD316 plasmid, pBHGloxΔE1 and 3Cre (Invitrogen, United States). The details have been previously published. The vector of interest was selected with polymerase chain reaction (PCR) amplification of inserted *Akt* gene using the following primer pair as show: F: 5'-GGGAATTCATGGGATGCGTGTGTAGC-3'; R: 5'-GGGGATCCTCAGGCCGTGCCGCTGGCCGAGTA-3'. The PCR thermal condition consisted of 94 ℃ 5 min, 30 cycles of 94 ℃ 30 s, 58 ℃ 30 s, 72 ℃ 1 min, and a final extension at 72 ℃ 5 min. The amplified gene was further confirmed with DNA sequencing (Sunbiotech Co., Ltd, ABi 3730, Beijing). adenoviral enhanced green fluorescent protein (Ad-EGFP) was purchased from Stratagene (CA, United States) and used as a control vector for tracking the distribution of virus after introduced to animal. The concentration of recombinant adenovirus was measured with tissue culture infective dose method as previously reported[24].

Plasmids were amplified in DH5a competent cells and purified using a commercial kit (Qiagen). Viruses were prepared in HEK293 cells (Qiagen, GmbH, Hilden, German), which contain the necessary gene for the virus package. Specifically, one day prior to the transfection, HEK293 cells were seeded at a concentration of 10×10^6 in 10 mL complete dulbecco's modified eagle medium and cultured overnight. Upon transfection, 1 mg/mL plasmid stock was taken and adjusted into a volume of 1600 μL, 150 μL calcium phosphate was added to the same tube, mixed well and incubated at room temperature for 20 min. The resultant mixture was slowly added into HEK293 cell culture. The dish was gently moved and swirled to allow the even distribution of virus in culture. Cells were further cultured for 16 h. The supernatant was discarded and replaced with fresh medium. After a 2-d culture, cells were examined under a fluorescence microscope (E80i, Nikon, Japan) for the green fluorescent protein (GFP^+) cells. Cells were harvested and lysed using freezing (-70 ℃) and thawing (37 ℃) twice. The resultant mixture was centrifuged at 10000 *g* for 10 min. The supernatant with virus was collected and stored at -70 ℃ for future infection of target cells.

Rat cirrhosis model and experiment groups

Fifty male rats (weight 220 ± 20 g) were purchased from Animal Center of Hunan Agriculture University (Hunan, China). Rats were housed under 25 ℃ ± 2 ℃ and a 12-h light/dark cycle in microisolater cages. Ten rats were randomly selected and used for the normal control. The remaining 40 rats were subjected to the induction of cirrhosis using CCl_4 as previously reported[25]. These rats were further randomly divided into 4 groups (n = 10 per

group). One group did not receive additional treatment, the other three groups received saline, Ad-EGFP, Ad-myr-HA-Akt (3.0×10^{11} vp in 1 mL saline), respectively, *via* tail vein injection at 2 wk after cirrhosis induction. The treatment was repeated at 6 wk. Accordingly, the experiment groups of rats were defined as follows: normal control, untreated cirrhosis, saline cirrhosis, EGFP cirrhosis and Akt cirrhosis. All surgical procedures were completed in accordance with the guidelines on the care and use of laboratory animals for research purposes by the Central South University Xiangya Medical School's Animal Care and Use Committee. Mice were anesthetized with chloral hydrate (*iv*) for all surgical procedures.

Hemodynamic and ultrasound parameters

Three days after treatment, five rats from each experimental groups (normal control group, untreated cirrhosis, saline cirrhosis, Ad-EGFP and Akt cirrhosis) were restricted from food with free access to water for 12 h. Rats were anesthetized by *iv* infusion of 2.5% chloral hydrate (50 gtt/min). Then, diameter (D) and peak velocity of blood flow (V) in portal was measured using ultrasound system (SIMENS Co., Ltd, Acuson Seguoia 512, Germany). The blood flow (Q) and congestion index (CI) was calculated using the following formulas: $Q = 0.57\pi D2/4V \times 60$, and $CI = \pi D2/4V$, respectively. Using a blood pressure device (RBP-1B, Sino-Japan friendship clinical medicine institute), the tail mean arterial pressure was recorded.

Analysis of liver function and histological changes

Eight weeks after cirrhosis induction, 1 mL of blood from each rat was collected from portal vein and subjected to analysis for alanine aminotransferase (ALT); aspartate aminotransferase (AST) and albumin (ALB) levels using a automatic biochemical analyzer (Spotchem SP4430, Arkray, Kyoto, Japan). Using aseptic techniques, laparotomy was performed, the portal vein was exposed, and portal vein pressure was measured with catheterization. Then rats were sacrificed and the right lobe of the liver was removed and stored in liquid nitrogen for future analysis. Liver tissue sections were subjected to HE staining for cellular and tissue structure as well as Van Gieson (VG) staining for collagen deposition. Hepatic hydroxyproline (Hyp), which is the main constituent of collagen protein, was used to estimate the degree of hepatic fibrosis, was measured with a commercial kit following manufacture's protocol (Jiancheng Biological product institute, Nanjing, China). The stained sections were examined and photographed under a microscope equipped with a digital photograph acquiring system (E80i, Nikon, Japan). Hyp and liver function markers were measured with an automatic machine (Beckman LX20, United States).

Flow cytometry analysis

Apoptosis of hepatic cells was detected using Annexin-V-fluorescein isothiocyanate (FITC)/Propidium iodide (PI) double staining and flow cytometry analysis. The cells were harvested and resuspended in Annexin-V binding buffer and further incubated with 5 μL of Annexin V-FITC and 10 μL of PI for 10 min at room temperature in the dark, followed by cytometric analysis (EPICS XL, Beckman Coulter, United States) within 30 min of staining. All experiments were performed in triplicate.

Western blotting

Using a protein extraction kit (Biovision, CA, United States), whole-cell extracts were prepared from frozen liver tissue. Protein concentration of the extracts was determined by the Bradford method with a kit purchased from Biovision (United States). Forty micrograms of protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and were electronically transferred onto a polyvinylidene difluoride membrane (Roche Diagnostics, Mannheim, Germany). The membrane was blocked in a standard western blotting procedure. Briefly, the membrane was blocked with 7.5% milk in tris-buffered-saline with tween (TBST) buffer [20 mmol/L Tris-HCl (pH 7.6), 137 mmol/L NaCl, 0.05% Tween 20], then probed with a primary antibody [anti-Akt, anti-phospho-Akt-Ser-473 (p-Akt) Akt, p-Akt, Fas, DR5 and HA, purchased from Cell Signaling Technology (Beverly, MA, United States). After washing with TBST buffer, the membrane was further incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:20000, KPL, United States). The protein bands were visualized using an enhanced chemiluminescence detection system, LumiGLO (KPL, United States). β-actin (NeoMarkers, Fremont, CA, United States) was used as a loading control and normalization reference for quantification.

Measurement of NO

Nitric acid reductase method was used to measure hepatic NO using a commercial kit (CST, United States), following the manufacture manual. Briefly, 1 g of rat liver tissue stored in the liquid nitrogen was thawed and homogenized at 4 ℃ in the saline at a concentration of 100 g/L. Following centrifuge (1000 r/min for 5 min), the supernatant was used for the nitric acid reductase reaction to measure product of NO_2^- and NO_3^-, which indirectly represent the level of NO.

Examination of transplanted GFP$^+$ cells

GFP+ cells in the host were examined at 8 wk after induction of cirrhosis. Mice was euthanized and infused with normal saline until the liver became pale. The liver, spleen, heart, lung, brain and kidney were collected, cryostat sectioned at a thickness of 2 μm, and examined for GFP$^+$ cells under a fluorescence microscope (E80i, Nikon, Japan). A fraction of the tissues from the above organs were also formalin-fixed, paraffin-embedded, continuously sectioned for H and E (hematoxylin and eosin) staining and histological analysis.

Statistical analysis

The SPSS program (version 12.0, SPSS Inc., United

Table 1 Portal vein diameter, peak velocity of blood flow, congestion index and blood flow in each experimental group

Group	*n*	D (mm)	V (cm/s)	CI	Q (mL/min)
Normal	5	1.13 ± 0.24	12.67 ± 0.64	0.0010 ± 0.0003	4.56 ± 1.86
Cirrhosis	20				
Untreated	5	1.81 ± 0.19[a]	10.13 ± 0.68[a]	0.0021 ± 0.0007[a]	9.69 ± 2.58[a]
Saline	5	1.83 ± 0.29[a]	10.06 ± 0.72[a]	0.0024 ± 0.0008[a]	9.32 ± 2.83[a]
EGFP	5	1.82 ± 0.27[a]	9.98 ± 0.77[a]	0.0020 ± 0.0006[a]	8.97 ± 2.45[a]
Akt	5	1.28 ± 0.32[c,e,g]	11.39 ± 0.63[c,e,g]	0.0013 ± 0.0004[c,e,g]	5.11 ± 2.30[c,e,g]

[a]$P < 0.05$ (*vs* Normal control); [c]$P < 0.05$ (*vs* Untreated group); [e]$P < 0.05$ (*vs* Saline group); [g]$P < 0.05$ [*vs* enhanced green fluorescent protein (EGFP) group]. No difference was detected among Untreated, Saline and EGFP groups. D: Diameter; V: Peak velocity of blood flow; CI: Congestion index; Q: Blood flow.

Table 2 Liver function parameter in each experiment group in each experimental group

Group	*n*	ALT (U/L)	AST (U/L)	ALB (g/L)	Hyp (μg/g)
Normal	5	23.5 ± 6.3	109.3 ± 6.1	33.1 ± 2.6	180.5 ± 12.5
Cirrhosis	20				
Untreated	5	277.6 ± 25.8[a]	380.5 ± 16.9[a]	22.7 ± 3.5[a]	375.2 ± 17.3[a]
Saline	5	290.7 ± 22.9[a]	368.9 ± 23.8[a]	24.7 ± 3.7[a]	393.8 ± 22.3[a]
EGFP	5	285.9 ± 27.3[a]	374.4 ± 26.7[a]	23.9 ± 2.9[a]	388.5 ± 9.8[a]
Akt	5	126.4 ± 5.8[a,c,e,g]	202.5 ± 9.5[a,c,e,g]	30.7 ± 4.8[a,c,e,g]	245.9 ± 15.6[a,c,e,g]

[a]$P < 0.05$ (*vs* Normal control); [c]$P < 0.05$ (*vs* Untreated group); [e]$P < 0.05$ (*vs* Saline group); [g]$P < 0.05$ [*vs* enhanced green fluorescent protein (EGFP) group]. No difference was detected among Untreated, Saline and EGFP groups. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALB: Albumin; Hyp: Hepatic hydroxyproline.

States) was used for statistical analysis. Quantitative data were expressed as mean ± SD. Student *t* test and/or one-way Analysis of variance was used for group comparisons. The differences were considered significant when $P < 0.05$.

RESULTS

Preparation of Ad-myr-HA-Akt virus and transfer to rats

As detailed in the method, the Ad-myr-HA-Akt virus was confirmed using PCR amplification of inserted Akt gene and sequencing for full length of inserted fragment, which was consistent with the sequence reported in the GenBank. The concentrations of Ad-myr-HA-Akt and Ad-EGFP virus used in the current study were 5.5×10^{11} vp/mL. The virus was introduced to animal through tail veil injection. According to the control vector with expression of green fluorescent protein (GFP), the distribution of virus was mainly in the liver (Figure 1).

Hemodynamic results of portal vein in different experimental groups

Five rats from each group (normal control group, untreated cirrhosis, saline cirrhosis, Ad-EGFP, and Akt cirrhosis groups) were subjected to measure portal vein diameter (D) and peak velocity of blood flow (V) and calculation of portal Q and CI (Figure 2). As shown in the Table 1, these parameters were significantly increased

Table 3 Portal vein pressure, mean arterial pressure and heart rate in each experiment group at the end of the treatment

Group	*n*	PVP (mmHg)	MAP (mmHg)	HR
Normal	5	8.96 ± 1.46	83.5 ± 9.8	323 ± 73
Cirrhosis	20			
Untreated	5	16.01 ± 1.32[a]	79.6 ± 14.2	339 ± 89
Saline	5	15.87 ± 1.40[a]	80.1 ± 11.5	282 ± 101
EGFP	5	15.65 ± 1.18[a]	82.9 ± 12.9	319 ± 78
Akt	5	9.23 ± 1.51[c,e,g]	88.5 ± 17.6	289 ± 96

[a]$P < 0.05$ (*vs* Normal control); [c]$P < 0.05$ (*vs* Untreated group); [e]$P < 0.05$ (*vs* Saline group); [g]$P < 0.05$ [*vs* enhanced green fluorescent protein (EGFP) group]. No difference was detected among Untreated, Saline and EGFP groups. PVP: Portal vein pressure; MAP: Mean arterial pressure; HR: Heart rate.

in untreated, saline and Ad-EGFP cirrhosis groups when compared to normal control. In contrast, these parameters in the Akt cirrhosis group were comparable to normal control group.

Ad-myr-HA-Akt virus was able to preserve liver function and reduce portal hypertension

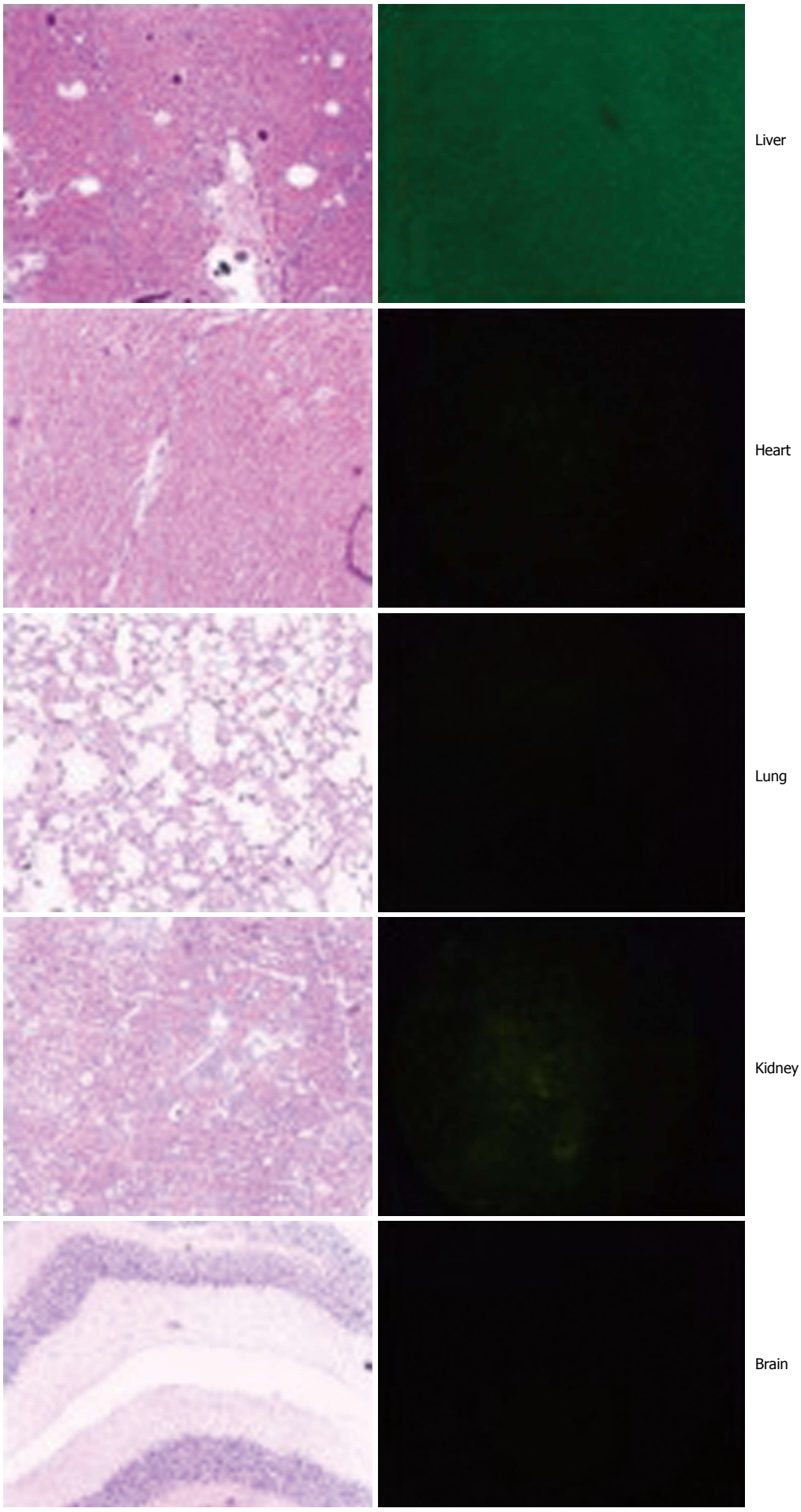
Eight weeks after cirrhosis induction, blood samples from portal vein were collected and subjected to the measurement of ALT, AST and ALB. As shown in the Table 2, compared to the normal control, the liver function was significantly impaired in the untreated, saline and Ad-EGFP cirrhosis groups. However, the Akt cirrhosis group showed significant improvement of liver function (lower levels of the four parameters) when compared to the untreated, saline and Ad-EGFP cirrhosis groups. Consistently, the Hyp level (Table 2) and portal vein pressure (Table 3) in Akt cirrhosis groups were also significantly lower than other cirrhosis groups (untreated, saline and Ad-EGFP). Of note, Mean arterial pressur and heart rate did not showed significant differences among the groups.

Ad-myr-HA-Akt virus significantly reduced the liver fibrosis

To determine whether the above observations were derived from the histological changes of liver, we examined cellular and tissue structure and collagen deposition using HE and VG staining, respectively (Figure 3). Our results indicated that Akt group has better preservation of histological structure and less fibrosis than other cirrhosis groups. Collectively, these data supported that Ad-myr-HA-AKT virus was efficient in inhibiting the development of cirrhosis induced by CCl_4.

Ad-myr-HA-Akt virus inhibited apoptosis of hepatocytes

To confirm the reduction of liver fibrosis was related to the reduction of apoptosis of hepatocytes, we doubly stained hepatocytes with PI and Annenin V to detect the apoptosis rate in each experimental group. As shown in Figure 4, the percentage of apoptotic cell was greatly less in Akt cirrhosis group than in other cirrhosis groups (2.5%-3.9% reduction). These results suggested that


Liver
Heart
Lung
Kidney
Brain

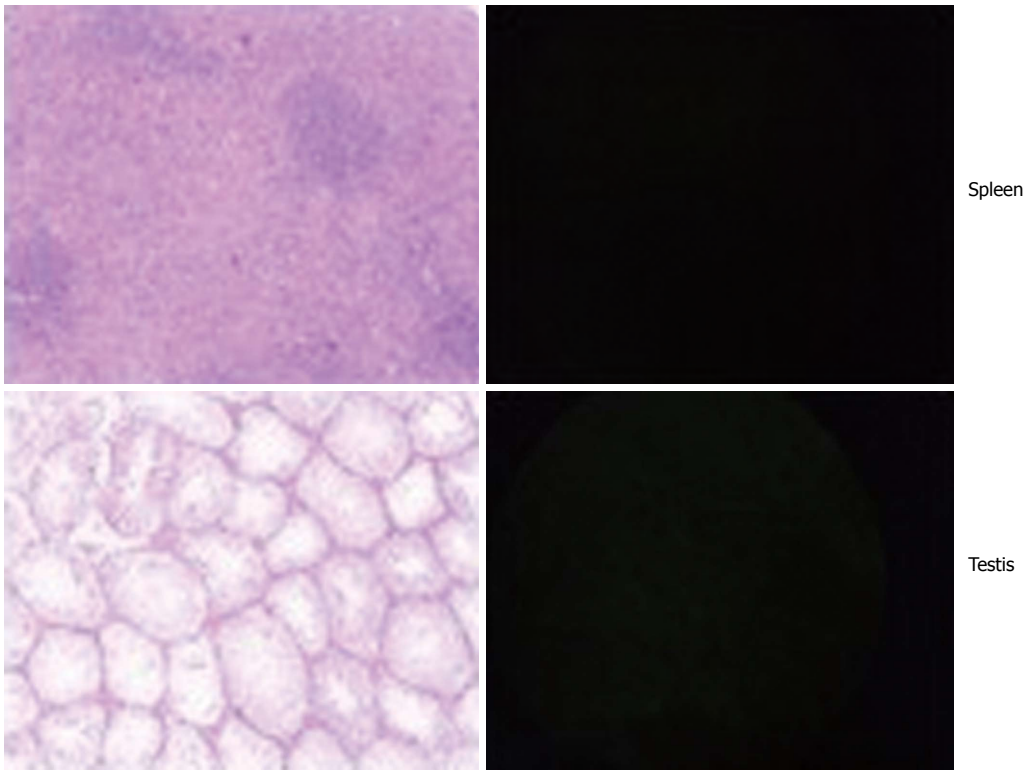


Figure 1 Tissue distribution of transferred viruses in the host. Representative micrographs from each organ (as designated) are shown for the presence of green fluorescent protein (GFP), which is a marker for the presence of the recombinant virus. GFP was primarily observed in the liver (top panel) and rarely present in other organs.

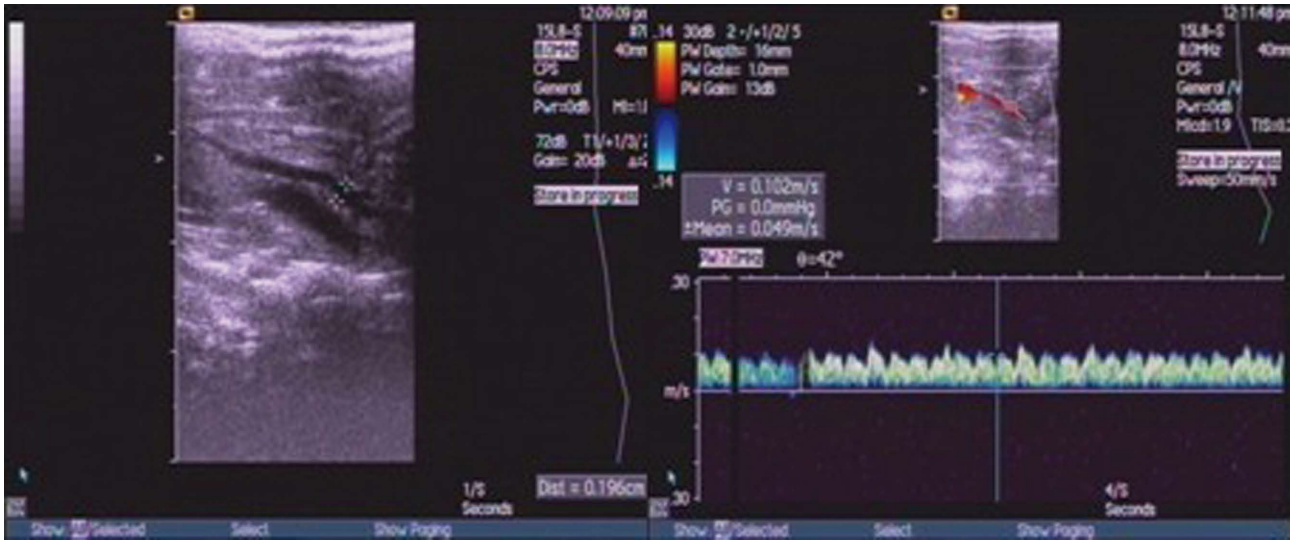


Figure 2 Measurement of hemodynamic parameter of portal vein. Representative graphs for identifying portal vein and measurement of hemodynamic parameters using ultrasound.

amelioration of liver function and fibrosis in Akt may be involved in the reduction of apoptosis of hepatocytes.

Ad-myr-HA-Akt virus inhibited apoptotic mediators

Next, to confirm the apoptosis, we measured the expression levels of apoptotic mediators using Western blotting. As shown in Figure 5A. Akt group showed positive HA tag and an increased level of phosphorylated Akt (p-Akt) as well as decreased levels of Fas. The levels of p-Akt and Fas are comparable in Akt cirrhosis group and normal control group. In contrast, Caspase-3 and Caspase-9 levels in Akt group were significantly lower than other cirrhosis groups (untreated, saline and Ad-EGFP groups). These results suggested that introduction of Ad-myr-HA-Akt virus resulted in the inhibition of Fas-mediated apoptotic pathway, which presumably led to the

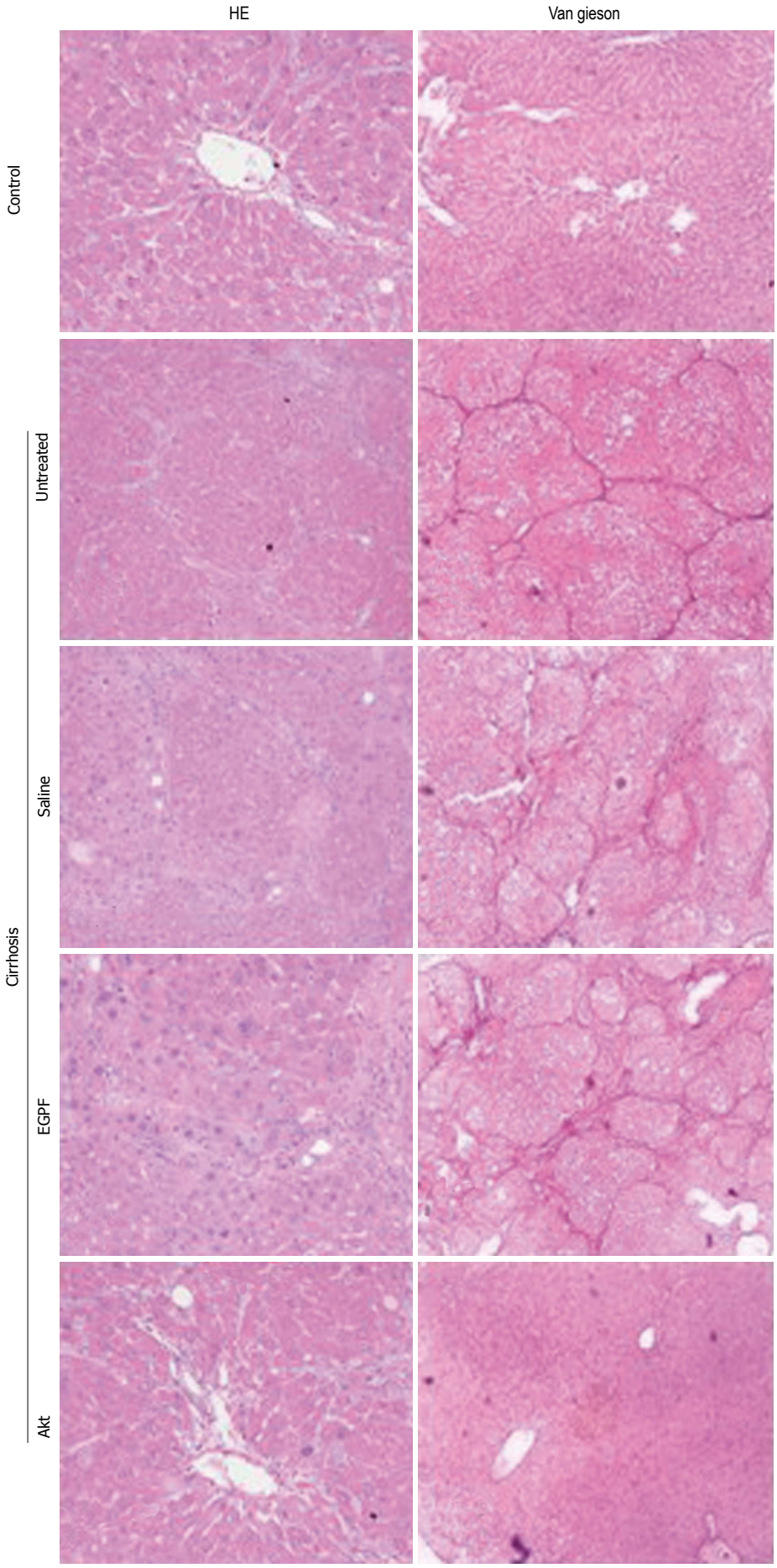


Figure 3 Histological changes in rat with cirrhosis induced by CCl_4. Livers from rats in each experimental group were collected 8 wk after CCl_4 treatment, sectioned, and subjected to HE and van gieson staining for tissue structure and collagen deposition. While cirrhosis groups without treatment or received saline or adenhanced green fluorescent protein treatment show structure disruption and nodule formation as well as remarkable deposition of collagen, cirrhosis group with Akt virus transfer show well preserved tissue structure and less collagen, both of which are comparable to normal control (magnification × 100).

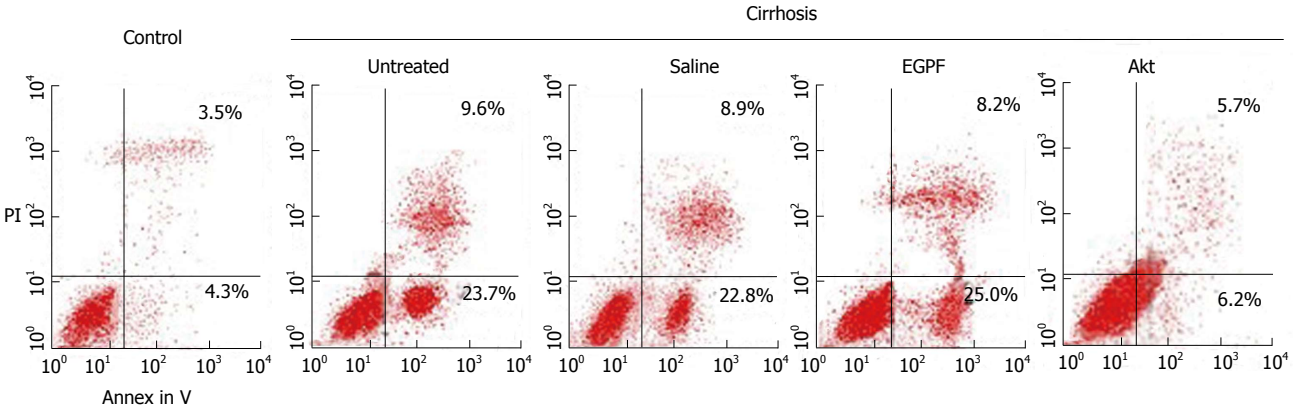


Figure 4 Flow cytometry analysis for hepatocyte apoptosis in each experimental group. Single hepatocytes from each experiment groups were prepared at 2 wk after CCl_4 treatment, and subjected to double staining of Propidium iodide (PI) and Annenin V to detect the dead and apoptotic cell. Representative data from each group are shown with designated name. Double positive cell with PI and Annenin V were considered as apoptotic dead cells and its percentage is listed in the right upper quadrant of the plot.

Table 4 NO content in liver tissue

Groups	*n*	NO (μmol/g)
Normal	5	2.53 ± 0. 61
Cirrhosis	20	
Untreated	5	1.20 ± 0.77[a]
Saline	5	1.03 ± 0.87[a]
EGFP	5	1.15 ± 0.58[a]
Akt	5	2.38 ± 0.67[c,e,g]

[a]$P < 0.05$ (*vs* normal control); [c]$P < 0.05$ (*vs* Untreated group); [e]$P < 0.05$ (*vs* Saline group); [g]$P < 0.05$ [*vs* enhanced green fluorescent protein (EGFP) group]. No difference was detected among Untreated, Saline and EGFP groups.

reduction of the apoptosis of hepatocytes.

Ad-myr-HA-Akt virus inhibited activation of hepatic stellate cell and increased the levels of eNOS activity and NO production

To understand the mechanism underlying the hepatocyte apoptosis and fibrosis of liver, we further measured two markers (DR5 and α-SMA) for the activation of HSC and the levels of eNOS and its phosphorylation status that is correlated to the NO production. Noticeable decrease of DR5 and α-SMA (Figure 5A) and increase of phosphorylated eNOS (Figure 5B) were observed in the Akt group when compared to other cirrhosis groups (untreated, saline and Ad-EGFP). As shown in Table 4, the NO level in liver was significantly higher in Akt group than other cirrhosis groups (untreated, saline and Ad-EGFP), which was consistent with the level of phosphorylated eNOS in these group. Collectively, these results suggested that introduction of Ad-myr-HA-Akt virus blocked the activation of HSC and maintained NO level, which may subsequently reduced liver fibrosis and blood vessel resistance following the damage from CCl_4.

DISCUSSION

Fas is one of the most important receptors on cell surface to mediate apoptosis[26-29]. It has been shown that FasL-Fas pathway is an important cascade leading to hepatocyte apoptosis, which in turn activates HSC that produce collagen[1-5]. Song *et al*[30] showed that block of Fas signaling pathway could inhibit the development of cirrhosis. Akt plays important roles in regulating cell survival through inhibiting Fas-mediated apoptosis[11,12]. In the current study, we utilized the constitutive expression of active form of Akt to block cirrhosis induced by CCl_4. The efficacy of this virus was firstly confirmed at the level of liver function. Furthermore, using multiple approaches, we examined whether the transfer of Akt *via* this virus in liver could result in the molecular alterations that favor the survival of hepatocyte and/or disfavor the fibrosis. Our data indicated that Ad-myr-HA-Akt virus was a useful tool to prevent CCl_4-induced cirrhosis in rat model.

Encouragingly, the data obtained at different levels, from function to histological changes, apoptosis rate of hepatocytes, activation of HSC, deposition of collagen, portal vein pressure and NO level, which were all consistently and collectively supported the hypothesis that introduction of Ad-myr-HA-Akt virus inhibits the development of cirrhosis. First, all measured parameters for liver function were consistent with reduced hepatocyte apoptosis: the introduction of Akt virus led to increased expression of Akt and its phosphorylation, decreased expression of apoptotic mediators (Caspase-9 and Caspase-3) and ultimately preserved liver functions (enzyme levels). Second, the portal vein pressure was consistent with the histological structure as well as NO levels. The introduction of Akt virus led to reduction of formation of liver nodules, portal vein pressures and increased level of NO, which is directly correlated with vasodilation. Third, the level of specific marker (Hyp) for the liver fibrosis was consistent with the amount of deposition of collagen and the expression of α-SMA and DR5, markers for activated HSC.

While the introduction of Ad-myr-HA-Akt virus led to the rescue of phosphorylated Akt level as well as in-

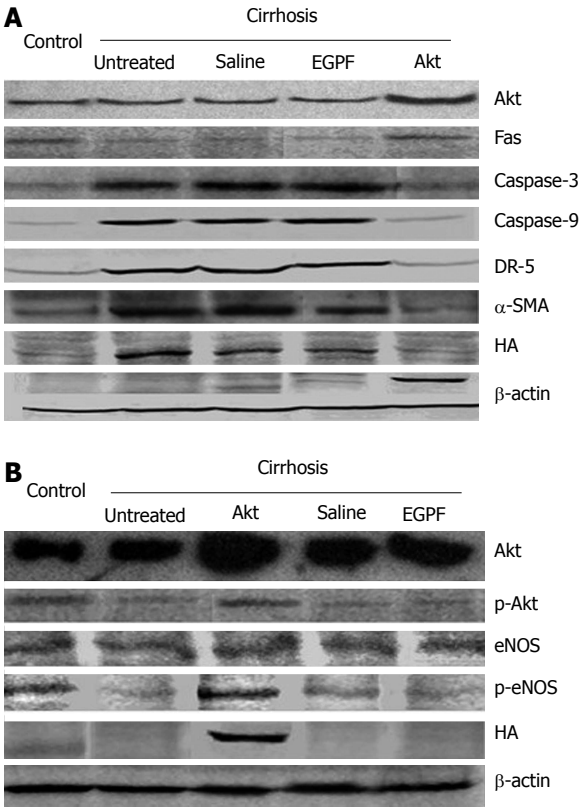


Figure 5 Western blotting analysis for apoptotic mediators and activation of hepatic stellate cells and endothelial nitric oxide synthase level. A: Apoptotic mediators and activation of HSC; Liver tissue from each group of rats were homogenized and subjected to measurement for the levels of Akt, phosphorylated Akt (p-Akt), Fas, Caspase-9 and Caspase-3, and HSC activation markers, DR5 and α-SMA, as designated in the figure. B: eNOS level; Liver tissues from each group of rats were homogenized and subjected to the measurements for Akt, pAkt, eNOS and phosphorylated eNOS (p-eNOS). Blotting of HA was used to confirm the expression of recombinant HA-Akt protein, and β-actin was used for the loading control. Data are representative of 3 experiments. HSC: Hepatic stellate cells; eNOS: Endothelial nitric oxide synthase.

hibition of apoptotic pathway in the liver of rat cirrhosis model. However, several issues remain to be addressed regarding the application of this virus for the treatment for cirrhosis. First, the efficacy of this form of virus in other type of cirrhosis, especially those chronically developed remains to be determined. In addition, regarding the development of the cirrhosis, it was thought that HSC activation plays a crucial role in producing collagen[1-5]. While we speculate that reduced HSC activation in Akt group may be a subsequent outcome of reduced hepatocyte apoptosis, it is unknown whether the introduction of Akt-virus has a direct effect on HSC activation. Furthermore, we observed that rats in the cirrhosis groups without treatment or received saline or Ad-EGFP treatment showed a noticeable reduction of phosphorylated Akt level, suggesting that the damage of hepatocytes following CCl_4 treatment may also be involved in the disruption of Akt signaling pathway. Lastly, one of the risks of the constitutive activation of Akt is the increased susceptibility to tumorgenesis. We could not conduct a long-term study to determine the time of virus clearance and evaluate the risk of tumor development. As future directions, we will further determine the application of this virus in other forms of cirrhosis and assess its side effects.

ACKNOWLEDGEMENTS

We thank Dr. Liwu Gou (Buffloa Roswell Park institute, New York State University, United States) for providing pcDNA3.1-myr-HA-Akt.

COMMENTS

Background

Massive apoptosis of hepatocytes result in activation of hepatic stellate cells (HSC), which produce collagen and ultimately lead to the fibrosis and cirrhosis. Inhibiting hepatic apoptosis is considered as an important strategy to prevent cirrhosis. Akt plays a crucial role in preventing Fas signaling-mediated hepatic apoptosis, and that over-expression of Akt was capable of preventing hepatic apoptosis.

Research frontiers

The progress in understanding mechanisms of cirrhosis brings the development of effective therapies closer to reality. Points of therapeutic intervention may include: (1) removing the injurious stimuli; (2) suppressing hepatic inflammation; (3) down-regulating stellate cell activation; and (4) promoting matrix degradation. The future prospects for effective treatment are more promising than ever for the millions of patients with chronic liver disease worldwide.

Innovations and breakthroughs

To date, there have been a number of studies regarding the therapeutic implication of hepatic cirrhosis. However, the research about the strategy to block apoptotic signaling pathway are limited. In this study, the authors created an Ad-myr-HA-Akt virus and determined the efficacy of this virus in inhibiting the development of cirrhosis induced by CCl_4 in rats. The authors confirmed that that introduction of Ad-myr-HA-Akt virus was not only able to ameliorate the liver cirrhosis but also to reduce the portal vein pressure.

Applications

These results could be the basis for further studies to understand the pathogenesis of hepatic cirrhosis. The conclusion suggest that Akt pathway may be a therapeutic target for human cirrhosis.

Terminology

HSC, also known as perisinusoidal cells or Ito cells are pericytes found in the perisinusoidal space of the liver. Substantial evidence now exists to recognize HSCs as the main matrix-producing cells in the process of liver cirrhosis. Liver injury of any etiology will ultimately lead to activation of HSCs, which undergo transdifferentiation to fibrogenic myofibroblast-like cells.

Peer review

Authors demonstrated that Akt improved liver histology and function and reduced portal venous pressure, liver apoptosis and collagen levels is useful for accurate understanding the progress of hepatic cirrhosis and also give a feasible target for the therapy of cirrhosis.

REFERENCES

1 **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]
2 **Friedman SL.** Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008; **134**: 1655-1669 [PMID: 18471545 DOI: 10.1053/j.gastro.2008.03.003]
3 **Mehal W,** Imaeda A. Cell death and fibrogenesis. *Semin Liver Dis* 2010; **30**: 226-231 [PMID: 20665375 DOI: 10.1055/s-0030-1255352]
4 **Forbes SJ,** Parola M. Liver fibrogenic cells. *Best Pract Res Clin Gastroenterol* 2011; **25**: 207-217 [PMID: 21497739 DOI: 10.1016/j.bpg.2011.02.006]
5 **Iwaisako K,** Brenner DA, Kisseleva T. What's new in liver

fibrosis? The origin of myofibroblasts in liver fibrosis. *J Gastroenterol Hepatol* 2012; **27** Suppl 2: 65-68 [PMID: 22320919 DOI: 10.1111/j.1440-1746.2011.07002.x]
6 **Li JT,** Liao ZX, Ping J, Xu D, Wang H. Molecular mechanism of hepatic stellate cell activation and antifibrotic therapeutic strategies. *J Gastroenterol* 2008; **43**: 419-428 [PMID: 18600385 DOI: 10.1007/s00535-008-2180-y]
7 **Kisseleva T,** Brenner DA. Anti-fibrogenic strategies and the regression of fibrosis. *Best Pract Res Clin Gastroenterol* 2011; **25**: 305-317 [PMID: 21497747 DOI: 10.1016/j.bpg.2011.02.011]
8 **Rockey DC**. Current and future anti-fibrotic therapies for chronic liver disease. *Clin Liver Dis* 2008; **12**: 939-962, xi [PMID: 18984475 DOI: 10.1016/j.cld.2008.07.011]
9 **Ruehl M,** Erben U, Kim K, Freise C, Dagdelen T, Eisele S, Trowitzsch-Kienast W, Zeitz M, Jia J, Stickel F, Somasundaram R. Extracts of Lindera obtusiloba induce antifibrotic effects in hepatic stellate cells via suppression of a TGF-beta-mediated profibrotic gene expression pattern. *J Nutr Biochem* 2009; **20**: 597-606 [PMID: 18824344 DOI: 10.1016/j.jnutbio.2008.06.003]
10 **Lin YL,** Lin CY, Chi CW, Huang YT. Study on antifibrotic effects of curcumin in rat hepatic stellate cells. *Phytother Res* 2009; **23**: 927-932 [PMID: 19152370 DOI: 10.1002/ptr.2764]
11 **Moumen A,** Ieraci A, Patané S, Solé C, Comella JX, Dono R, Maina F. Met signals hepatocyte survival by preventing Fas-triggered FLIP degradation in a PI3k-Akt-dependent manner. *Hepatology* 2007; **45**: 1210-1217 [PMID: 17464994 DOI: 10.1002/hep.21604]
12 **Hatano E,** Brenner DA. Akt protects mouse hepatocytes from TNF-alpha- and Fas-mediated apoptosis through NK-kappa B activation. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G1357-G1368 [PMID: 11705740]
13 **Imamura Y**, Ishikawa S, Sato N, Karashima R, Hirashima K, Hiyoshi Y, Nagai Y, Koga Y, Hayashi N, Watanabe M, Yamada G, Baba H. Adenoviral oncolytic suicide gene therapy for a peritoneal dissemination model of gastric cancer in mice. *Ann Surg Oncol* 2010; **17**: 643-652 [PMID: 20012217 DOI: 10.1245/s10434-009-0852-3.]
14 **Liu RY,** Zhu YH, Zhou L, Zhao P, Li HL, Zhu LC, Han HY, Lin HX, Kang L, Wu JX, Huang W. Adenovirus-mediated delivery of interferon-γ gene inhibits the growth of nasopharyngeal carcinoma. *J Transl Med* 2012; **10**: 256 [PMID: 23272637 DOI: 10.1186/1479-5876-10-256]
15 **Nie ZL,** Pan YQ, He BS, Gu L, Chen LP, Li R, Xu YQ, Gao TY, Song GQ, Hoffman AR, Wang SK, Hu JF. Gene therapy for colorectal cancer by an oncolytic adenovirus that targets loss of the insulin-like growth factor 2 imprinting system. *Mol Cancer* 2012; **11**: 86 [PMID: 23171475 DOI: 10.1186/1476-4598-11-86]
16 **Zhu H,** Zhou W, Hu J, Huang Z, Lao W, Huang X, He C. Suppressing the growth of rectal cancer xenografts derived from patient tumors by an adenovector expressing small hairpin RNA targeting Bcl-XL. *J Gene Med* 2012; **14**: 761-768 [PMID: 23169477 DOI: 10.1002/jgm.2681]
17 **Yamanaka M,** Tada Y, Kawamura K, Li Q, Okamoto S, Chai K, Yokoi S, Liang M, Fukamachi T, Kobayashi H, Yamaguchi N, Kitamura A, Shimada H, Hiroshima K, Takiguchi Y, Tatsumi K, Tagawa M. E1B-55 kDa-defective adenoviruses activate p53 in mesothelioma and enhance cytotoxicity of anticancer agents. *J Thorac Oncol* 2012; **7**: 1850-1857 [PMID: 23154556 DOI: 10.1097/JTO.0b013e3182725fa4]
18 **Fukui H,** Wong HT, Beyer LA, Case BG, Swiderski DL, Di Polo A, Ryan AF, Raphael Y. BDNF gene therapy induces auditory nerve survival and fiber sprouting in deaf Pou4f3 mutant mice. *Sci Rep* 2012; **2**: 838 [PMID: 23150788 DOI: 10.1038/srep00838]
19 **Kinoshita K,** Iimuro Y, Fujimoto J, Inagaki Y, Namikawa K, Kiyama H, Nakajima Y, Otogawa K, Kawada N, Friedman SL, Ikeda K. Targeted and regulable expression of transgenes in hepatic stellate cells and myofibroblasts in culture and in vivo using an adenoviral Cre/loxP system to antagonise hepatic fibrosis. *Gut* 2007; **56**: 396-404 [PMID: 16956920 DOI: 10.1136/gut.2005.085704]
20 **Ahn M,** Gamble A, Witting SR, Magrisso J, Surendran S, Obici S, Morral N. Vector and helper genome rearrangements occur during production of helper-dependent adenoviral vectors. *Hum Gene Ther Methods* 2013; **24**: 1-10 [PMID: 23249343 DOI: 10.1089/hgtb.2012.198]
21 **Pei Z,** Kondo S, Kanegae Y, Saito I. Copy number of adenoviral vector genome transduced into target cells can be measured using quantitative PCR: application to vector titration. *Biochem Biophys Res Commun* 2012; **417**: 945-950 [PMID: 22202173 DOI: 10.1016/j.bbrc.2011.12.016]
22 **Chiyo T,** Sekiguchi S, Hayashi M, Tobita Y, Kanegae Y, Saito I, Kohara M. Conditional gene expression in hepatitis C virus transgenic mice without induction of severe liver injury using a non-inflammatory Cre-expressing adenovirus. *Virus Res* 2011; **160**: 89-97 [PMID: 21645560 DOI: 10.1016/j.virusres.2011.05.019]
23 **Winter LE,** Barenkamp SJ. Construction and immunogenicity of recombinant adenovirus vaccines expressing the HMW1, HMW2, or Hia adhesion protein of nontypeable Haemophilus influenzae. *Clin Vaccine Immunol* 2010; **17**: 1567-1575 [PMID: 20685934 DOI: 10.1128/CVI.00115-10]
24 **Segura MM**, Monfar M, Puig M, Mennechet F, Ibanes S, Chillón M. A real-time PCR assay for quantification of canine adenoviral vectors. *J Virol Methods* 2010; **163**: 129-136 [PMID: 19751766 DOI: 10.1016/j.jviromet.2009.09.010]
25 **Hua J,** Qiu de K, Li JQ, Li EL, Chen XY, Peng YS. Expression of Toll-like receptor 4 in rat liver during the course of carbon tetrachloride-induced liver injury. *J Gastroenterol Hepatol* 2007; **22**: 862-869 [PMID: 17504260 DOI: 10.1111/j.1440-1746.2007.04896.x]
26 **Akazawa Y,** Gores GJ. Death receptor-mediated liver injury. Semin *Liver Dis* 2007; **27**: 327-338 [PMID: 17979070 DOI: 10.1055/s-2007-991510]
27 **Okano H,** Shiraki K, Inoue H, Kawakita T, Yamanaka T, Deguchi M, Sugimoto K, Sakai T, Ohmori S, Fujikawa K, Murata K, Nakano T. Cellular FLICE/caspase-8-inhibitory protein as a principal regulator of cell death and survival in human hepatocellular carcinoma. *Lab Invest* 2003; **83**: 1033-1043 [PMID: 12861043 DOI: 10.1097/01.LAB.0000079328.76631.28]
28 **Reinehr R,** Häussinger D. CD95 death receptor and epidermal growth factor receptor (EGFR) in liver cell apoptosis and regeneration. *Arch Biochem Biophys* 2012; **518**: 2-7 [PMID: 22182753 DOI: 10.1016/j.abb.2011.12.004]
29 **Guicciardi ME,** Gores GJ. Apoptosis as a mechanism for liver disease progression. *Semin Liver Dis* 2010; **30**: 402-410 [PMID: 20960379 DOI: 10.1055/s-0030-1267540]
30 **Song E,** Lee SK, Wang J, Ince N, Ouyang N, Min J, Chen J, Shankar P, Lieberman J. RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat Med* 2003; **9**: 347-351 [PMID: 12579197 DOI: 10.1038/nm828]

P- Reviewers: Abdel-Raheem IT, Trinder D **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.v19.i43.7788

World J Gastroenterol 2013 November 21; 19(43): 7788-7794
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Insulin-like growth factor-1 induces lymphangiogenesis and facilitates lymphatic metastasis in colorectal cancer

Zhen-Jun Li, Xiao-Jiang Ying, Hong-Liang Chen, Ping-Jiang Ye, Zhi-Liang Chen, Gang Li, Hua-Feng Jiang, Jiang Liu, Shu-Zhen Zhou

Zhen-Jun Li, Xiao-Jiang Ying, Hong-Liang Chen, Ping-Jiang Ye, Zhi-Liang Chen, Gang Li, Hua-Feng Jiang, Jiang Liu, Shu-Zhen Zhou, Department of Colorectal Surgery, Shaoxing People's Hospital, Shaoxing Hospital of Zhejiang University, Shaoxing 312000, Zhejiang Province, China

Author contributions: Li ZJ and Ying XJ designed the research and wrote the manuscript; Chen HL, Ye PJ, Li G and Jiang HF performed the experiments; Liu J and Zhou SZ performed the statistical analysis; Liu J and Chen ZL collected the clinical data and provided some useful comments during the study; all authors have read and approved the final manuscript.

Supported by Technological Research Project for Public Welfare from Science and Technology Department of Zhejiang Province, No. 2010C33099

Correspondence to: Xiao-Jiang Ying, MD, Department of Colorectal Surgery, Shaoxing People's Hospital, Shaoxing Hospital of Zhejiang University, No. 568, Zhongxing North Street, Shaoxing 312000, Zhejiang Province, China. lzdx2003@163.com
Telephone: +86-575-88229151 Fax: +86-575-88228823
Received: July 24, 2013 Revised: September 18, 2013
Accepted: September 29, 2013
Published online: November 21, 2013

Abstract

AIM: To investigate the expression of insulin-like growth factor-1 (IGF-1)/insulin-like growth factor-1 receptor (IGF-1R) in colorectal cancer (CRC) tissues and to analyze their correlation with lymphangiogenesis and lymphatic metastasis.

METHODS: Immunohistochemistry was used to evaluate IGF-1 and IGF-1R expression and lymphatic vessel density (LVD) in 40 CRC specimens. The correlation between IGF-1/IGF-1R and LVD was investigated. Effects of IGF-1 on migration and invasion of CRC cells were examined using transwell chamber assays. A LoVo cell xenograft model was established to further detect the role of IGF-1 in CRC lymphangiogenesis *in vivo*.

RESULTS: Elevated IGF-1 and IGF-1R expression in CRC tissues was correlated with lymph node metastasis (r = 0.715 and 0.569, respectively, $P < 0.05$) and tumor TNM stage (r = 0.731 and 0.609, $P < 0.05$). A higher LVD was also found in CRC tissues and was correlated with lymphatic metastasis (r = 0.405, $P < 0.05$). A positive correlation was found between LVD and IGF-1R expression (r = 0.437, $P < 0.05$). Transwell assays revealed that IGF-1 increased the migration and invasion of CRC cells. *In vivo* mouse studies showed that IGF-1 also increased LVD in LoVo cell xenografts.

CONCLUSION: IGF-1/IGF-1R signaling induces tumor-associated lymphangiogenesis and contributes to lymphatic metastasis of CRC.

Key words: Colorectal cancer; Insulin-like growth factor-1; Insulin-like growth factor-1 receptor; Lymphangiogenesis; Lymphatic metastasis

Core tip: Insulin-like growth factor-1 (IGF-1) and its receptor, insulin-like growth factor-1 receptor (IGF-1R), are frequently overexpressed in many types of tumors including colorectal cancer. A recent study (Björndahl *et al*, 2003) showed that both IGF-1 and IGF-2 could potently stimulate lymphatic vessel growth in the mouse cornea. However, equivalent evidence on IGF-1 in solid tumors is lacking. Here, we show that IGF-1/IGF-1R signaling induces tumor-associated lymphangiogenesis *in vivo* and contributes to lymphatic metastasis of colorectal cancer. Findings from the present study provide further evidence to support the involvement of IGF-1/IGF-1R signaling in lymphangiogenesis in solid tumors.

Li ZJ, Ying XJ, Chen HL, Ye PJ, Chen ZL, Li G, Jiang HF, Liu J,

Zhou SZ. Insulin-like growth factor-1 induces lymphangiogenesis and facilitates lymphatic metastasis in colorectal cancer. *World J Gastroenterol* 2013; 19(43): 7788-7794 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7788.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7788

INTRODUCTION

Lymphatic metastasis is one of the most common metastatic routes of colorectal cancer (CRC). When cancer spreads, it is much harder to treat successfully. The presence or absence of lymph node involvement is one of the most important factors that determine the long-term outcome of cancer patients[1-3]. There is a complex process that a tumor cell must go through before metastasis can occur[4,5]. Excessive formation of new lymphatic vessels in CRC is a key step in metastatic progression[5,6]. However, the factors triggering lymphangiogenesis and the detailed molecular mechanisms are poorly understood.

Insulin-like growth factor-1 (IGF-1) and its receptor (IGF-1R) are frequently overexpressed in many types of tumors including CRC[7-10]. Increasing evidence suggests that amplified IGF-1/IGF-1R signaling not only is associated with an increased relative risk for cancer development, but also contributes to cancer cell survival, invasion, metastasis and resistance to chemotherapeutic drugs[7,10]. In addition to their roles in the development and progression of cancer, the IGF-1/IGF-1R signaling system can also induce lymphangiogenesis[11,12]. In a recent study, Björndahl *et al*[11] reported that both IGF-1 and IGF-2 could potently stimulate lymphatic vessel growth in the mouse cornea. However, direct evidence showing the involvement of IGF-1/IGF-1R signaling in lymphangiogenesis in solid tumors is lacking.

In the present study, we found that IGF-1 and IGF-1R were significantly overexpressed in CRC tissues compared with adjacent normal tissues using an immunohistochemical (IHC) assay. Using a lymphatic endothelial-specific antibody marker D2-40[3,13], we found that lymphatic vessel density (LVD) was significantly higher in CRC tissues. The levels of IGF-1, IGF-1R and LVD were all significantly correlated with lymphatic metastasis. In addition, a positive correlation was found between LVD and IGF-1R. These results suggest that the IGF-1/IGF-1R axis might promote lymph node metastasis of CRC by induction of lymphangiogenesis. To further explore its role in lymphangiogenesis, we created a LoVo cell (a human colon cancer cell line) xenograft model and showed that IGF-1 treatment resulted in an increase in the LVD *in vivo*. Together, our findings demonstrate that IGF-1/IGF-1R signaling can induce lymphangiogenesis in CRC and may facilitate lymphatic metastasis in CRC patients.

MATERIALS AND METHODS

Tissue samples

Forty CRC and adjacent normal tissue samples were obtained from randomly selected patients undergoing surgical resection without preoperative neoadjuvant chemoradiotherapy between January 2011 and June 2011 at Shaoxing People's Hospital. Their average age was 68.5 years (range, 44 to 83 years). Of these patients, 9 had well-differentiated adenocarcinoma, 20 had moderately differentiated adenocarcinoma, and 11 had poorly differentiated adenocarcinoma. Twenty-five patients had stage Ⅰ-Ⅱ disease and 15 had stage Ⅲ-Ⅳ disease according to the tumor-node-metastasis (TNM) classification defined by Union for International Cancer Control (UICC)[14]. D2 radical resection was performed in 3 patients and D3 radical resection was performed in 37 patients. The number of lymph nodes resected was 9-22.

Immunohistochemical staining

Tissue sections were stained using the Envision System (DakoCytomation, Carpinteria, CA, United States) according to manufacturer's instructions. Mouse monoclonal antibodies against human IGF-1, IGF-1R, or D2-40 were obtained from Abcam (Cambridge, United Kingdom). Intensity of immunostaining signals was evaluated in 8 fields under a light microscope (Olympus Optical, Tokyo, Japan). Statistical analysis was carried out in accordance with a previous study[15]. In short, total staining of IGF-1 and IGF-1R were scored as the product of the staining intensity (on a scale of 0-3: negative = 0, weak = 1, moderate = 2, strong = 3) and the percentage of cells stained (0 = 0%, 1 = 1%-25%, 2 = 26%-50%, 3 = 51%-100%), which resulted in a scale of 0-9. Two independent pathologists scored each sample without prior knowledge of the patient's clinical information and outcome.

Cell culture and IGF-1 treatment

The human CRC cell line LoVo was obtained from the American Type Culture Collection and maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin in a 5% CO_2 incubator at 37 ℃. For IGF-1 treatment, media were replaced with fresh serum-free medium containing 50 ng/mL IGF-1 (PeproTech) when cells were grown to 30% confluency. An IGF-1 stock solution was prepared in PBS, and thus, PBS in serum-free medium was used as a control.

Migration and invasion analysis

The migration and invasion of CRC cells were examined using non-coated or matrigel-coated transwell culture chambers (8 μm pore size, Corning, NY, United States) 48 h after IGF-1 treatment. For migration assays, 1×10^5 cells were seeded in the top non-coated chamber and incubated at 37 ℃ for 8 h. For invasion assays, 5×10^4 cells were seeded in the top Matrigel-coated (BD Biosciences, Bedford, MA, United States) chambers and incubated at 37 ℃ for 24 h. In both assays, cells were suspended in serum-free Roswell Park Memorial Institute (RPMI)-1640 medium in the upper chamber, and the lower chamber was filled with RPMI-1640 medium containing 5% FBS. After incubation, the top chambers were wiped with cot-

Table 1 Expression of insulin-like growth factor-1 and insulin-like growth factor-1 receptor in colorectal cancer tissues

	n	IGF-1	*P* value	IGF-1R	*P* value
Tissue type			0.001		0.002
Adjacent normal tissues	40	1.4 ± 0.6		1.3 ± 1.2	
Cancer tissue	40	4.5 ± 2.5		4.7 ± 2.7	
Differentiation			0.012		0.001
Well	9	3.0 ± 1.4		2.1 ± 0.9	
Moderately	20	4.3 ± 2.6		4.6 ± 2.4	
Poorly	11	6.1 ± 2.1		6.6 ± 2.1	
Lymph node metastasis			0.003		0.002
Yes	15	6.8 ± 2.0		6.3 ± 2.2	
No	25	3.1 ± 1.5		3.5 ± 2.2	

IGF-I: Insulin-like growth factor-1; IGF-I R: Insulin-like growth factor-1 receptor.

ton wool to remove the non-migratory or non-invasive cells. Cells on the underside of the membrane were fixed in methanol for 30 min, stained with 0.1% crystal violet, and counted under a microscope (Eclipse TS100, Nikon, Japan).

Xenograft and IGF-1 treatment

Four-week-old BALB/c nude mice (18-22 g) were used in this study. LoVo cells in the logarithmic phase of growth were adjusted to a density of 5×10^7/mL, and 0.2 mL of cell suspension was subcutaneously injected into the flank of the mice. On the second day after LoVo cell transplantation, the mice were randomized into either a treatment or a control group, with ten mice in each group. Referring to previous reports[16-19], IGF-1 (50 μg/kg) was administered every day by intraperitoneal injection in the treatment group. The mice in the control group received the same amount of normal saline. Three weeks after treatment, the cancer tissues were dissected from the nude mice for measurement of LVD.

Measurement of LVD

LVD in tissue sections was quantitatively analyzed using the EnVision system with the specific lymphatic endothelial cell marker, D2-40, which allows for accurate discrimination of lymphatic vessels from blood vessels[3,13]. Five fields with the most abundant lymphatic regions (hot spots) were chosen by light microscopy at 40 × magnification. The LVD was then assessed by counting all stained vessels at 200 × magnification. Single immunoreactive endothelial cells or endothelial cell clusters separated from other microvessels were counted as a vessel according to previous procedures[20]. The average number of lymphatic vessels in five selected fields was taken as the LVD on the slide.

Statistical analysis

All statistical analyses were performed using SPSS (version 12.0, SPSS Inc, United States). Student's *t* test test, Mann-Whitney *U* test and χ^2 test were used for statistical analyses. Spearman's rank correlation was used for correlation analysis. *P* values < 0.05 were considered statistically significant.

RESULTS

Up-regulation of IGF-1 and IGF-1R in colorectal cancer

Immunohistochemistry analysis was performed to examine the expression of IGF-1 and IGF-1R in CRC samples. IGF-1 and IGF-1R were weakly or negatively stained in tumor-adjacent normal tissues. In contrast, they were weakly or moderately stained in well-differentiated CRC tissues, and moderately or strongly stained in moderately and poorly differentiated CRC tissues (Table 1 and Figure 1). As expected, expression of IGF-1 was detected primarily in the cytoplasm and IGF-1R was detected on the membrane (Figure 1). Furthermore, the expression of IGF-1 and IGF-1R was correlated with lymph node metastasis (r = 0.715 and 0.569, respectively; $P < 0.05$) and TNM stage (r = 0.731 and 0.609, respectively; $P < 0.05$). These results indicate that elevated expression of IGF-1/IGF1R parallels CRC progression.

Correlation between LVD and expression of IGF-1/IGF-1R

It was reported that LVD is an indicator of lymphangiogenesis[3,21]. D2-40 is a specific lymphatic endothelial cell marker for the evaluation of LVD in human cancers[3,13]. Using IHC, we found that D2-40 was localized in the cytoplasm and membrane of lymphatic endothelial cells (Figure 2). LVD was significantly higher in CRC tissue as compared to non-tumor tissue (11.45 ± 3.03 *vs* 3.25 ± 1.28, $P < 0.05$). LVD in tumor tissues with lymph node metastasis was higher than that in tissues without metastasis (12.67 ± 4.09 *vs* 10.72 ± 1.90, $P < 0.05$) (Table 2 and Figure 2). Lymphatic vessel invasion was also detected in CRC tissue (Figure 2B and C). There was a significant correlation between LVD and lymphatic metastasis ($R = 0.405$, $P < 0.05$). In addition, a statistically significant correlation was also found between LVD and IGF-1R ($R = 0.437$, $P < 0.05$). These results suggest that the IGF-1/IGF-1R system might promote lymph node metastasis of CRC through induction of lymphangiogenesis.

IGF-1 increases migration and invasion of LoVo cells

In transwell migration assays, a significantly higher number of cells migrated through the chamber membrane after IGF-1 treatment (196.4 ± 21.6 *vs* 96.4 ± 11.2, $P < 0.05$, Figure 3A). Similar results were obtained in the transwell invasion assays. Cells which migrated through the matrigel and chamber membrane increased in the IGF-1-treated group (163.6 ± 19.4 *vs* 72.5 ± 9.1, $P < 0.05$, Figure 3B). These results indicated that IGF-1 increased the migration and invasion potential of CRC cells.

Effects of IGF-1 on LVD of LoVo cell xenografts in nude mice

To assess whether IGF-1 affected lymphangiogenesis in CRC *in vivo*, LoVo cells were implanted subcutaneously

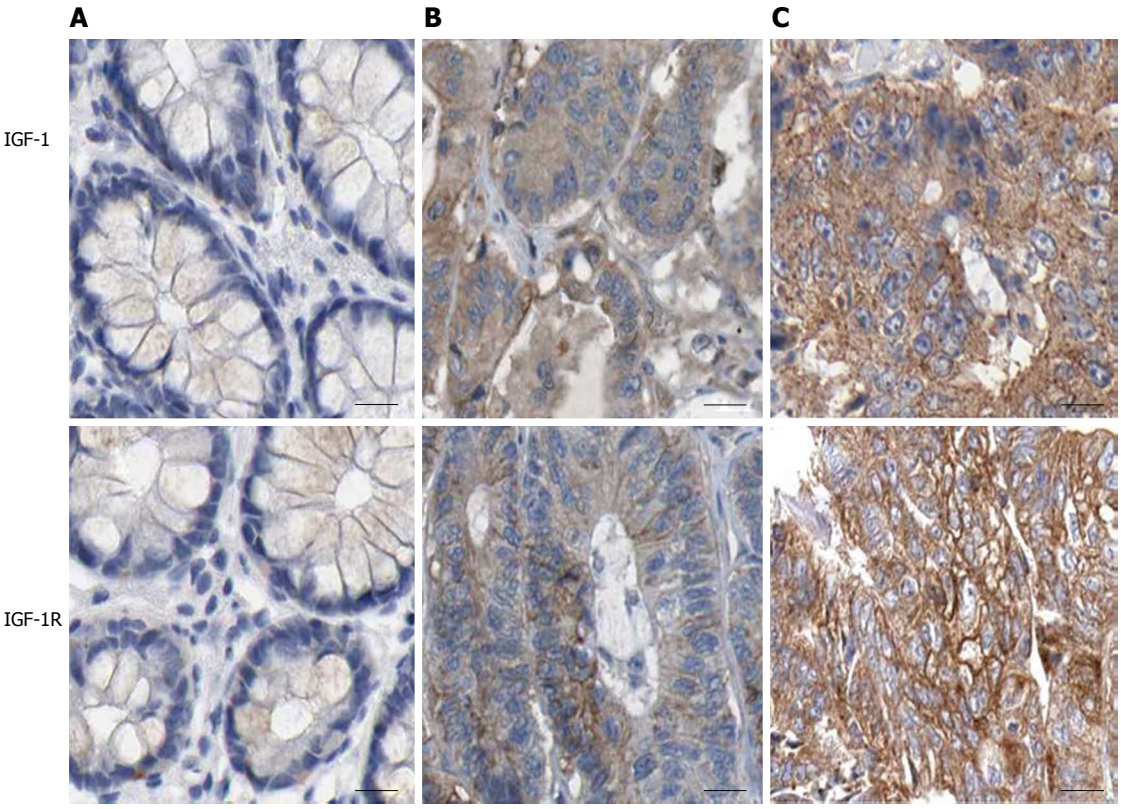


Figure 1 Immunohistochemical staining of insulin-like growth factor-1 and insulin-like growth factor-1 receptor in colorectal cancer tissues. A: Tumor-adjacent normal tissues; B: Moderately differentiated CRC and C, poorly differentiated CRC (× 200). IGF-1: Insulin-like growth factor-1. IGF-I R: Insulin-like growth factor-1 receptor; CRC: Colorectal cancer.

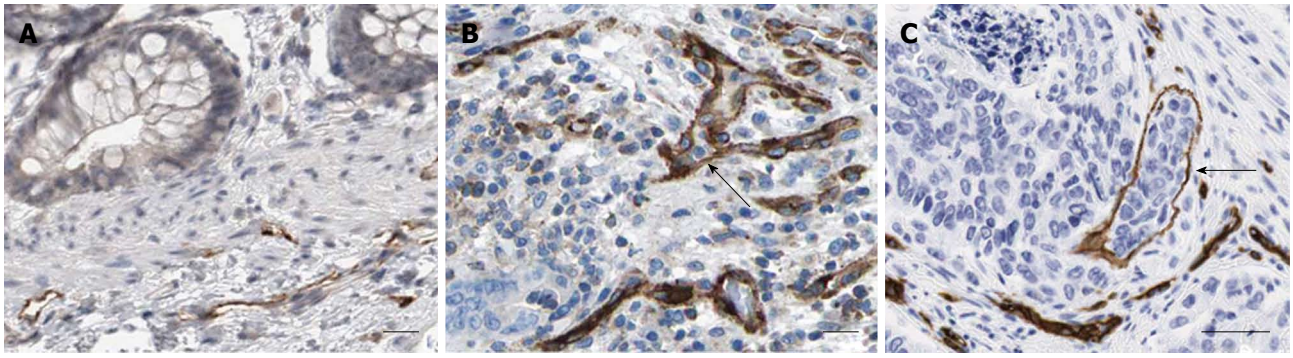


Figure 2 Morphological features of D2-40 positive lymphatic vessels in colorectal cancer. A: Tumor-adjacent normal tissues; B and C: Colorectal cancer tissues (× 200). Lymphatic vessel invasion of tumor cells was also detected in B and C (black arrow). IGF-1: Insulin-like growth factor-1.

Table 2 Lymphatic vessel density in colorectal cancer tissues

	n	LVD	*P* value
Tissue type			0.001
Adjacent normal tissues	40	3.3 ± 1.3	
Cancer tissue	40	11.5 ± 3.0	
Lymph node metastasis			0.032
Yes	15	12.7 ± 4.1	
No	25	10.7 ± 3.9	

LVD: Lymphatic vessel density.

into nude mice. Three weeks after daily IGF-1 or vehicle administration, tumor tissues were removed and measured. IHC analysis using anti-D2-40 antibodies revealed that LVD of LoVo cell xenografts was significantly higher in the IGF-1 group than in the control group (10.7 ± 3.3 *vs* 6.4 ± 2.9, $P < 0.05$, Figure 4).

DISCUSSION

The importance of lymph node metastasis in cancer pro-

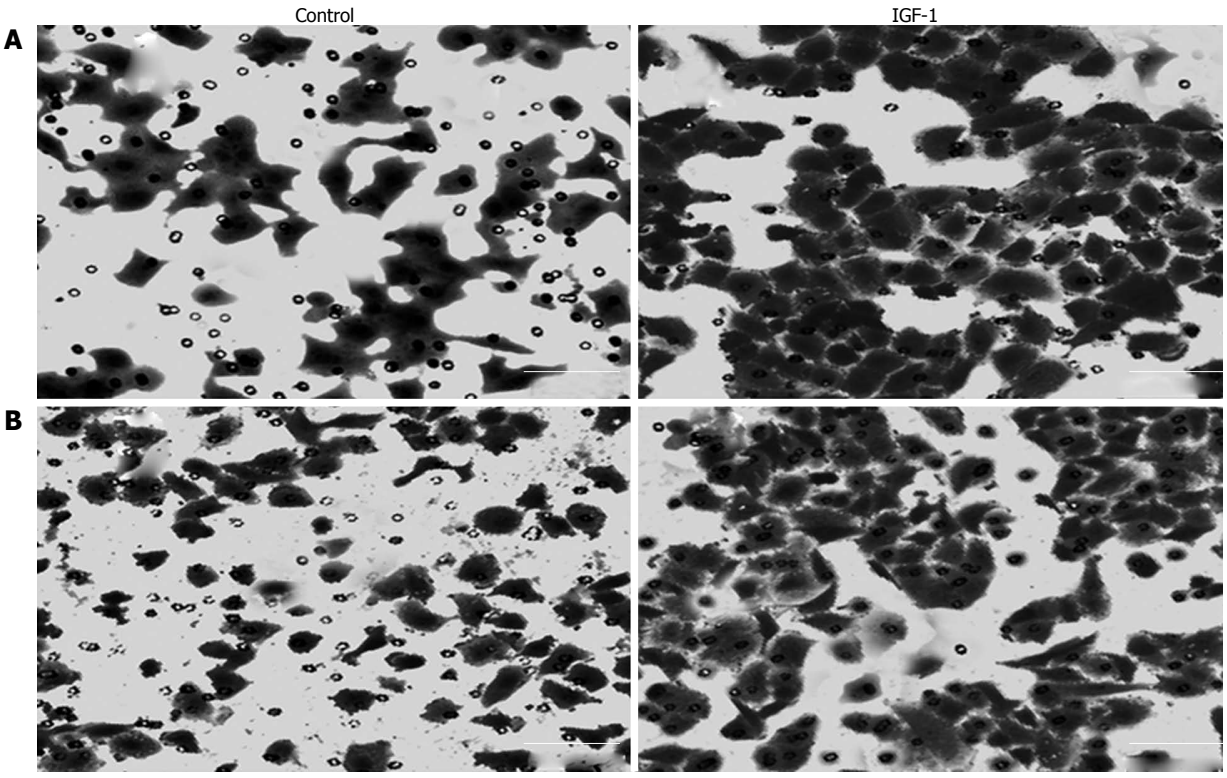


Figure 3 Effect of insulin-like growth factor-1 on the migration and invasion of LoVo cells after 48 h treatment. A: Cell migration; B: Cell invasion (× 200). IGF-1: Insulin-like growth factor-1.

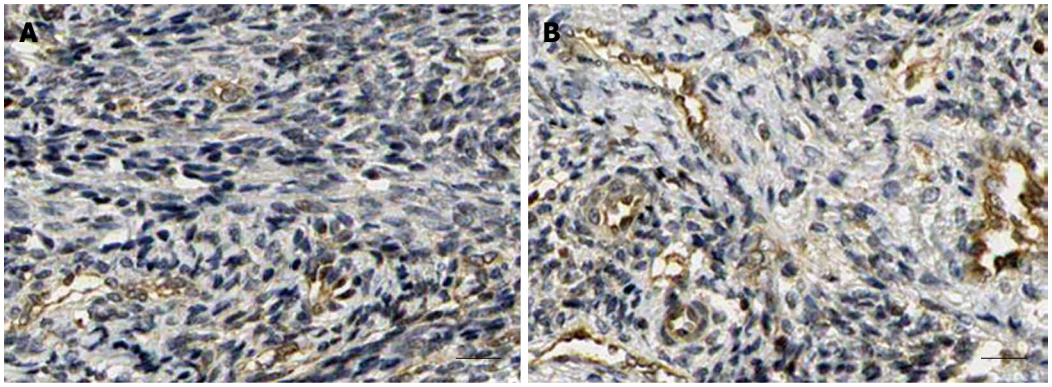


Figure 4 Effect of insulin-like growth factor-1 on lymphatic vessel density in LoVo cell xenografts in nude mice after treatment with insulin-like growth factor-1. A: Control group; B: 50 μg/kg IGF-1 group (× 200). IGF-1: Insulin-like growth factor-1; IGF-I R: Insulin-like growth factor-1 receptor.

gression has been well established and is considered one of the most important prognostic factors[1,2,22,23]. Recently, there is growing evidence that tumor lymphangiogenesis plays an important role in this process[5,6]. However, the detailed molecular mechanisms that regulate lymphangiogenesis remain largely unknown. In the present study, we have shown that IGF-1/IGF-1R signaling can induce tumor-associated lymphangiogenesis, and therefore, may facilitate further lymphatic metastasis of CRC.

IGF-1 and its receptor IGF-1R are frequently expressed in many solid tumors and have been implicated in cancer metastasis[7,24-26]. In 1999, Hakam *et al*[27] showed that IGF1-R plays a role in the evolution of colorectal adenoma to carcinoma and favors the metastasis of CRC. In a recent study, Wu *et al*[28] reported that IGF-1 is critical in activating and sustaining an inflammatory response in the liver which is needed for CRC hepatic metastasis. In the present study, we found that IGF-1 and IGF-1R protein expression was elevated in CRC tissues, and their expression was correlated with clinical stage and lymphatic metastasis, which is consistent with the reported data.

Lymphangiogenesis, the formation of new lymphatic vessels, is a key process in lymphatic invasion and metastasis[29]. Numerous studies have shown that LVD is an indicator of lymphangiogenesis and represents a tool to determine the metastatic risk of neoplasia[3,21,29]. A study by Cacchi *et al*[1] revealed that CRC could induce lymphangiogenesis and a higher LVD could increase the

capability of cancer cells to invade the lymphatic system. In another study, Matsumoto *et al*[30] reported that both LVD and lymphatic vessel invasion were related to an adverse outcome in CRC. Similar results were obtained in this study. In addition, lymphatic vessel invasion was also detected in CRC tissue by IHC staining. Moreover, a statistically significant correlation was found between LVD and the level of IGF-1R in CRC tissue. This suggests that IGF-1/IGF-1R signaling probably facilitates metastasis of CRC by inducing tumor-associated lymphangiogenesis.

Recently, a number of lymphangiogenic factors which stimulate the proliferation of lymphatic vessels and lymphangiogenesis have been identified, including vascular endothelial growth factor (VEGF)-A, VEGF-C, VEGF-D, fibroblast growth factor 2, and platelet-derived growth factors[11,31]. Most of these lymphangiogenic factors were shown to be directly or indirectly regulated by IGF-1[11,12,32]. These findings prompted us to hypothesize that IGF-1/IGF-1R signaling promotes lymphangiogenesis. In 2005, Björndahl *et al*[11] reported that both IGF-1 and IGF-2 could stimulate lymphatic vessel growth in the mouse cornea. However, to date, it is still unclear whether a similar correlation exists in solid tumors. Here, we showed that IGF-1 could also induce lymphangiogenesis in CRC. The spread of metastasis then occurs through the new lymphatic vessel system in the tumor.

Tumor lymphangiogenesis and metastasis to lymph nodes are a complex process and a number of growth factors are involved in these events. IGFs are particularly interesting regulators due to their multiple roles in promoting tumor growth[33-35]. Here, the *in vivo* studies provided direct evidence that the IGF-1/IGF-1R axis can also induce lymphangiogenesis in solid tumors. However, so far, it is not known whether the IGF-1/IGF-1R axis directly acts on lymphatic endothelial cells to induce lymphangiogenesis or indirectly *via* other growth factor/receptor systems. In addition, the detailed mechanisms underlying lymphangiogenesis associated with the IGF-1/IGF-1R system are largely unknown. Therefore, additional efforts are warranted to study the relationship between IGF-1/IGF-1R and lymphangiogenesis in cancer.

In summary, we found that the IGF-1/IGF-1R system can induce tumor-associated lymphangiogenesis and facilitate lymphatic metastasis of CRC. Findings from the present study provide further evidence supporting the involvement of IGF-1/IGF-1R signaling in lymphangiogenesis in solid tumors. In addition, we also found that increased expression of IGF-1 and IGF-1R is correlated with tumor differentiation in human CRC. Our findings and others suggest that the IGF-1/IGF-1R axis plays critical and diverse roles in promoting tumor progression. Taken together, the IGF-1/IGF-1R axis is a potentially useful target for the treatment of cancer and metastasis.

COMMENTS

Background

Lymphangiogenesis plays an important role in lymphatic metastasis. Insulin-like growth factor-1 (IGF-1) and insulin-like growth factor-1 receptor (IGF-1R) are frequently overexpressed in many types of tumors including colorectal cancer. In addition to their role in the development and progression of cancer, the IGF-1/IGF-1R signaling system can also induce lymphangiogenesis.

Research frontiers

A recent study (Björndahl *et al*, 2003) showed that both IGF-1 and IGF-2 potently stimulated lymphatic vessel growth in the mouse cornea. However, equivalent evidence in solid tumors is lacking.

Innovations and breakthroughs

The authors of this paper demonstrate that the IGF-1/IGF-1R system can induce tumor-associated lymphangiogenesis in colorectal cancer (CRC) and contributes to its lymphatic metastasis, thus providing a novel mechanism for lymphangiogenesis in solid tumors.

Applications

The findings of this study are of value in further explaining the molecular mechanisms of lymphangiogenesis in CRC. The IGF-1/IGF-1R axis may be a potential target for the treatment of cancer and metastasis.

Terminology

IGF-1 is a hormone similar in molecular structure to insulin. It plays an important role in child growth and continues to have anabolic effects in adults. The role of IGF-1 in promoting cancer has been investigated for many years. Increasing evidence suggests that IGF-1 not only is associated with an increased relative risk for the development of cancer, but also contributes to cancer cell survival, invasion, metastasis and resistance to chemotherapeutic drugs.

Peer review

The authors examined the expression of IGF-1/IGF-1R in CRC tissues and its correlation with lymphangiogenesis and lymphatic metastasis. The study revealed that IGF-1/IGF-1R signaling system induces lymphangiogenesis in CRC and contributes to lymphatic metastasis of CRC. The results are interesting and provide further evidence that IGF-1/IGF-1R signaling is involved in lymphangiogenesis in solid tumors.

REFERENCES

1 **Cacchi C**, Arnholdt HM, Jähnig H, Anthuber M, Probst A, Oruzio DV, Märkl B. Clinical significance of lymph vessel density in T3 colorectal carcinoma. *Int J Colorectal Dis* 2012; **27**: 721-726 [PMID: 22228115 DOI: 10.1007/s00384-011-1373-7]

2 **Gao J**, Knutsen A, Arbman G, Carstensen J, Frånlund B, Sun XF. Clinical and biological significance of angiogenesis and lymphangiogenesis in colorectal cancer. *Dig Liver Dis* 2009; **41**: 116-122 [PMID: 19038587 DOI: 10.1016/j.dld.2008.07.315]

3 **Holmqvist A**, Gao J, Adell G, Carstensen J, Sun XF. The location of lymphangiogenesis is an independent prognostic factor in rectal cancers with or without preoperative radiotherapy. *Ann Oncol* 2010; **21**: 512-517 [PMID: 19889620 DOI: 10.1093/annonc/mdp486]

4 **Yokota J**. Tumor progression and metastasis. *Carcinogenesis* 2000; **21**: 497-503 [PMID: 10688870 DOI: 10.1093/carcin/21.3.497]

5 **Wang TB**, Chen ZG, Wei XQ, Wei B, Dong WG. Serum vascular endothelial growth factor-C and lymphoangiogenesis are associated with the lymph node metastasis and prognosis of patients with colorectal cancer. *ANZ J Surg* 2011; **81**: 694-699 [PMID: 22295309 DOI: 10.1111/j.1445-2197]

6 **Nagahashi M**, Ramachandran S, Rashid OM, Takabe K. Lymphangiogenesis: a new player in cancer progression. *World J Gastroenterol* 2010; **16**: 4003-4012 [PMID: 20731013 DOI: 10.3748/wjg.v16.i32.4003]

7 **Fürstenberger G**, Senn HJ. Insulin-like growth factors and cancer. *Lancet Oncol* 2002; **3**: 298-302 [PMID: 12067807 DOI: 10.1016/S1470-2045(02)00731-3]

8 **Hermani A**, Shukla A, Medunjanin S, Werner H, Mayer D. Insulin-like growth factor binding protein-4 and -5 modulate ligand-dependent estrogen receptor-α activation in breast cancer cells in an IGF-independent manner. *Cell Signal* 2013; **25**: 1395-1402 [PMID: 23499909 DOI: 10.1016/j.cellsig.2013.02.018]

9 **Neuhouser ML**, Platz EA, Till C, Tangen CM, Goodman PJ, Kristal A, Parnes HL, Tao Y, Figg WD, Lucia MS, Hoque A, Hsing AW, Thompson IM, Pollak M. Insulin-like growth factors and insulin-like growth factor-binding proteins and prostate cancer risk: results from the prostate cancer prevention trial. *Cancer Prev Res* (Phila) 2013; **6**: 91-99 [PMID: 23315596 DOI: 10.1158/1940-6207.CAPR-12-0250]

10 **Baserga R**, Peruzzi F, Reiss K. The IGF-1 receptor in cancer biology. *Int J Cancer* 2003; **107**: 873-877 [PMID: 14601044 DOI: 10.1002/ijc.11487]

11 **Björndahl M**, Cao R, Nissen LJ, Clasper S, Johnson LA, Xue Y, Zhou Z, Jackson D, Hansen AJ, Cao Y. Insulin-like growth factors 1 and 2 induce lymphangiogenesis in vivo. *Proc Natl Acad Sci USA* 2005; **102**: 15593-15598 [PMID: 16230630 DOI: 10.1073/pnas.0507865102]

12 **Da MX**, Wu Z, Tian HW. Tumor lymphangiogenesis and lymphangiogenic growth factors. *Arch Med Res* 2008; **39**: 365-372 [PMID: 18375246 DOI: 10.1016/j.arcmed.2007.12.005]

13 **Yan G**, Zhou XY, Cai SJ, Zhang GH, Peng JJ, Du X. Lymphangiogenic and angiogenic microvessel density in human primary sporadic colorectal carcinoma. *World J Gastroenterol* 2008; **14**: 101-107 [PMID: 18176970 DOI: 10.3748/wjg.14.101]

14 **Sobin LH**, Gospodarowicz MK, Wittekind C, editors. TNM classification of malignant tumors. 7th ed. Weinheim, Germany: Wiley, 2009

15 **Kreisberg JI**, Malik SN, Prihoda TJ, Bedolla RG, Troyer DA, Kreisberg S, Ghosh PM. Phosphorylation of Akt (Ser473) is an excellent predictor of poor clinical outcome in prostate cancer. *Cancer Res* 2004; **64**: 5232-5236 [PMID: 15289328 DOI: 10.1158/0008-5472.CAN-04-0272]

16 **Rinaldi C**, Bott LC, Chen KL, Harmison GG, Katsuno M, Sobue G, Pennuto M, Fischbeck KH. Insulinlike growth factor (IGF)-1 administration ameliorates disease manifestations in a mouse model of spinal and bulbar muscular atrophy. *Mol Med* 2012; **18**: 1261-1268 [PMID: 22952056]

17 **Murali SG**, Brinkman AS, Solverson P, Pun W, Pintar JE, Ney DM. Exogenous GLP-2 and IGF-I induce a differential intestinal response in IGF binding protein-3 and -5 double knockout mice. *Am J Physiol Gastrointest Liver Physiol* 2012; **302**: G794-G804 [PMID: 22281475 DOI: 10.1152/ajpgi.00372.2011]

18 **Zacharakis E**, Demetriades H, Pramateftakis MG, Lambrou I, Zacharakis E, Zaraboukas T, Koliakos G, Kanellos I, Betsis D. Effect of IGF-I on healing of colonic anastomoses in rats under 5-FU treatment. *J Surg Res* 2008; **144**: 138-144 [PMID: 17640667 DOI: 10.1016/j.jss.2007.03.045]

19 **Zacharakis E**, Demetriades H, Kanellos D, Sapidis N, Zacharakis E, Mantzoros I, Kanellos I, Koliakos G, Zaraboukas T, Topouridou K, Betsis D. Contribution of insulin-like growth factor I to the healing of colonic anastomoses in rats. *J Invest Surg* 2007; **20**: 9-14 [PMID: 17365402 DOI: 10.1080/08941930601126074]

20 **Schoppmann SF**, Birner P, Stöckl J, Kalt R, Ullrich R, Caucig C, Kriehuber E, Nagy K, Alitalo K, Kerjaschki D. Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. *Am J Pathol* 2002; **161**: 947-956 [PMID: 12213723 DOI: 10.1016/S0002-9440(10)64255-1]

21 **Barresi V**, Reggiani-Bonetti L, Di Gregorio C, De Leon MP, Barresi G. Lymphatic vessel density and its prognostic value in stage I colorectal carcinoma. *J Clin Pathol* 2011; **64**: 6-12 [PMID: 20947870 DOI: 10.1136/jcp.2010.083550]

22 **Yuan Y**, Li MD, Hu HG, Dong CX, Chen JQ, Li XF, Li JJ, Shen H. Prognostic and survival analysis of 837 Chinese colorectal cancer patients. *World J Gastroenterol* 2013; **19**: 2650-2659 [PMID: 23674872 DOI: 10.3748/wjg.v19.i17.2650]

23 **Lee SR**, Kim HO, Son BH, Yoo CH, Shin JH. Prognostic factors associated with long-term survival and recurrence in pancreatic adenocarcinoma. *Hepatogastroenterology* 2013; **60**: 358-362 [PMID: 23574658 DOI: 10.5754/hge12727]

24 **Hiraga T**, Myoui A, Hashimoto N, Sasaki A, Hata K, Morita Y, Yoshikawa H, Rosen CJ, Mundy GR, Yoneda T. Bone-derived IGF mediates crosstalk between bone and breast cancer cells in bony metastases. *Cancer Res* 2012; **72**: 4238-4249 [PMID: 22738911 DOI: 10.1158/0008-5472.CAN-11-3061]

25 **Huang F**, Xu LA, Khambata-Ford S. Correlation between gene expression of IGF-1R pathway markers and cetuximab benefit in metastatic colorectal cancer. *Clin Cancer Res* 2012; **18**: 1156-1166 [PMID: 22294722 DOI: 10.1158/1078-0432.CCR-11-1135]

26 **Zhang C**, Hao L, Wang L, Xiao Y, Ge H, Zhu Z, Luo Y, Zhang Y, Zhang Y. Elevated IGFIR expression regulating VEGF and VEGF-C predicts lymph node metastasis in human colorectal cancer. *BMC Cancer* 2010; **10**: 184 [PMID: 20459642 DOI: 10.1186/1471-2407-10-184]

27 **Hakam A**, Yeatman TJ, Lu L, Mora L, Marcet G, Nicosia SV, Karl RC, Coppola D. Expression of insulin-like growth factor-1 receptor in human colorectal cancer. *Hum Pathol* 1999; **30**: 1128-1133 [PMID: 10534157 DOI: 10.1016/S0046-8177(99)90027-8]

28 **Wu Y**, Brodt P, Sun H, Mejia W, Novosyadlyy R, Nunez N, Chen X, Mendoza A, Hong SH, Khanna C, Yakar S. Insulin-like growth factor-I regulates the liver microenvironment in obese mice and promotes liver metastasis. *Cancer Res* 2010; **70**: 57-67 [PMID: 20048072 DOI: 10.1158/0008-5472.CAN-09-2472]

29 **Lin M**, Ma SP, Lin HZ, Ji P, Xie D, Yu JX. Intratumoral as well as peritumoral lymphatic vessel invasion correlates with lymph node metastasis and unfavourable outcome in colorectal cancer. *Clin Exp Metastasis* 2010; **27**: 123-132 [PMID: 20195706 DOI: 10.1007/s10585-010-9309-0]

30 **Matsumoto K**, Nakayama Y, Inoue Y, Minagawa N, Katsuki T, Shibao K, Tsurudome Y, Hirata K, Nagata N, Itoh H. Lymphatic microvessel density is an independent prognostic factor in colorectal cancer. *Dis Colon Rectum* 2007; **50**: 308-314 [PMID: 17164964 DOI: 10.1007/s10350-006-0792-y]

31 **Stefansson IM**, Salvesen HB, Akslen LA. Vascular proliferation is important for clinical progress of endometrial cancer. *Cancer Res* 2006; **66**: 3303-3309 [PMID: 16540684 DOI: 10.1158/0008-5472.CAN-05-1163]

32 **Hirakawa S**, Kodama S, Kunstfeld R, Kajiya K, Brown LF, Detmar M. VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. *J Exp Med* 2005; **201**: 1089-1099 [PMID: 15809353 DOI: 10.1084/jem.20041896]

33 **Arcaro A**. Targeting the insulin-like growth factor-1 receptor in human cancer. *Front Pharmacol* 2013; **4**: 30 [PMID: 23525758 DOI: 10.3389/fphar.2013.00030]

34 **Bruchim I**, Werner H. Targeting IGF-1 signaling pathways in gynecologic malignancies. *Expert Opin Ther Targets* 2013; **17**: 307-320 [PMID: 23294364 DOI: 10.1517/14728222.2013.749863]

35 **Martin JL**, Baxter RC. Signalling pathways of insulin-like growth factors (IGFs) and IGF binding protein-3. *Growth Factors* 2011; **29**: 235-244 [PMID: 21895551 DOI: 10.3109/08977194.2011.614237]

P- Reviewers: Da MX, Lee SY, Natsugoe S, Shibata MA
S- Editor: Cui XM **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.v19.i43.7795

World J Gastroenterol 2013 November 21; 19(43): 7795-7803
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

BRIEF ARTICLE

Identification of Annexin A1 protein expression in human gastric adenocarcinoma using proteomics and tissue microarray

Zhi-Qiang Zhang, Xiu-Juan Li, Gui-Tao Liu, Xiang-Yang Zhang, Yu Xia, Hao Wen

Zhi-Qiang Zhang, Xiang-Yang Zhang, Yu Xia, Department of Gastroenterology, the First Affiliated Hospital, Xinjiang Medical University, Urumqi 830011, Xinjiang Uyghur Autonomous Region, China
Xiu-Juan Li, Department of Pathophysiology, Collage of Preclinical Medicine, Xinjiang Medical University, Urumqi 830011, Xinjiang Uyghur Autonomous Region, China
Gui-Tao Liu, Occupational Diseases Prevention and Treatment Institution, the Fifth Affiliated Hospital, Xinjiang Medical University, Urumqi 830011, Xinjiang Uyghur Autonomous Region, China
Hao Wen, Department of Liver and Laparoscopic Surgery, Digestive and Vascular Centre, the First Affiliated hospital, Xinjiang Medical University, Urumqi 830011, Xinjiang Uyghur Autonomous Region, China
Author contributions: Zhang ZQ and Li XJ designed this study, performed the major experiments, analyzed the data and wrote the manuscript; Liu GT, Zhang XY and Xia Y participated in the performance of experiments and statistical analyses; and Wen H designed the study together and critically reviewed the manuscript.
Supported by National Natural Science Foundation of China, No. 81001101; Natural Science Foundation of Xinjiang Uygur Autonomous Region, China, No.2010211B20
Correspondence to: Hao Wen, Professor, Department of Liver and Laparoscopic Surgery, Digestive and Vascular Centre, the First Affiliated Hospital, Xinjiang Medical University, No. 137 Liyushan South Road, Urumqi 830011, Xinjiang Uyghur Autonomous Region, China. zhangzhiqiang011@126.com
Telephone: +86-991-4324139 Fax: +86-991-4324139
Received: June 30, 2013 Revised: August 23, 2013
Accepted: September 15, 2013
Published online: November 21, 2013

Abstract

AIM: To study the differential expression of Annexin A1 (ANXA1) protein in human gastric adenocarcinoma. This study was also designed to analyze the relationship between ANXA1 expression and the clinicopathological parameters of gastric carcinoma.

METHODS: Purified gastric adenocarcinoma cells (GAC) and normal gastric epithelial cells (NGEC) were obtained from 15 patients with gastric cancer by laser capture microdissection. All of the peptide specimens were labeled as $^{18}O/^{16}O$ after trypsin digestion. Differential protein expressions were quantitatively identified between GAC and NGEC by nanoliter-reverse-phase liquid chromatography-mass/mass spectrometry (nano-RPLC-MS/MS). The expressions of ANXA1 in GAC and NGEC were verified by western blot analysis. The tissue microarray containing the expressed ANXA1 in 75 pairs of gastric carcinoma and paracarcinoma specimens was detected by immunohistochemistry (IHC). The relationship between ANXA1 expression and clinicopathological parametes of gastric carcinoma was analyzed.

RESULTS: A total of 78 differential proteins were identified. Western blotting revealed that ANXA1 expression was significantly upregulated in GAC (2.17/1, $P < 0.01$). IHC results showed the correlations between ANXA1 protein expression and the clinicopathological parameters, including invasive depth (T stage), lymph node metastasis (N stage), distant metastasis (M stage) and tumour-lymph node metastasis stage ($P < 0.01$). However, the correlations between ANXA1 protein expression and the remaining clinicopathological parameters, including sex, age, histological differentiation and the size of tumour were not found ($P > 0.05$).

CONCLUSION: The upregulated ANXA1 expression may be associated with carcinogenesis, progression, invasion and metastasis of GAC. This protein could be considered as a biomarker of clinical prognostic prediction and targeted therapy of GAC.

Key words: Gastric cancer; Annexin A1 protein; Pro-

teomics; Tissue microarray; Immunohistochemistry

Core tip: The anti-inflammatory protein Annexin A1 (ANXA1) mediates various important physiological and pathophysiological processes. Evidence has shown that ANXA1 is related to the development and progression of human multi-tumours. However, the ANXA1 expression in gastric adenocarcinoma of Chinese patients and the relationship between this protein and its clinicopathological parameters remain unclear. In the present study, the ANXA1 expression in gastric adenocarcinoma of Chinese patients was investigated by proteomics and western blot analysis. Authors examined 75 pairs of gastric adenocarcinoma and paracarcinoma tissues by tissue microarray to determine the presence of ANXA1 by immunohistochemistry. They found that ANXA1 expression was upregulated and involved in human gastric adenocarcinoma invasion and metastasis. Our findings suggested that ANXA1 might be used as a valuable biomarker in clinical diagnosis, prognostic prediction and targeted therapy of gastric cancer.

Zhang ZQ, Li XJ, Liu GT, Zhang XY, Xia Y, Wen H. Identification of Annexin A1 protein expression in human gastric adenocarcinoma using proteomics and tissue microarray. *World J Gastroenterol* 2013; 19(43): 7795-7803 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7795.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7795

INTRODUCTION

Gastric cancer (GC) is a common digestive tract cancer because of the lack of early diagnosis strategies; a previous study revealed that GC cases are usually diagnosed when the disease is at an advanced stage[1]. On the basis of metastasis, recurrence and other causes, the treatment and prognosis of GC remain poor. Therefore, effective biomarkers of GC should be determined; the mechanism of incidence and development should also be investigated to promote early diagnosis, effective treatment and prognosis improvement of GC.

Annexin A1 (ANXA1) is a key member of the A subfamily and belongs to the multi-gene family of Annexins. ANXA1 exhibits calcium-mediated phospholipid binding properties and participates in many physiological and pathological processes. Further studies have shown that ANXA1 is abnormally expressed in various tumours. This abnormal expression is closely related to tumourigenesis, development, invasion and metastasis of human tumours. The expression of ANXA1 in tumours is tissue specific; for instance, a low ANXA1 expression is observed in oesophageal squamous cell carcinoma[2], whereas a high expression is found in colorectal cancer[3]. The relationship between ANXA1 expression in GC and GC invasion as well as metastasis remains unclear. ANXA1 also exhibits a low expression in GC and negatively correlates with invasion and metastasis[4]. However, other studies have revealed opposite results[5,6].

We performed laser capture microdissection (LCM) to investigate the relation of ANXA1 expression in GC to the clinical parameters and to obtain purified gastric adenocarcinoma cells (GAC) and normal gastric epithelial cells (NGEC). $^{18}O/^{16}O$ was used to label the digested peptides in the mixture of GAC and NGEC. Nanoliter-reverse-phase liquid chromatography-mass/mass spectrometry (nano-RPLC-MS/MS) was performed to identify and quantify the differentially expressed proteins. Nano-RPLC-MS/MS was also conducted to validate the results of proteomics. To verify the differential protein expression of ANXA1, we performed western blot. Immunohistochemistry (IHC) was performed to detect the expression of ANXA1 in 75 pairs of tissue microarray of GC tissues and paracarcinoma tissues. This study aimed to analyze the correlations of ANXA1 with clinical pathological parameters, including age, gender, differentiation degree, metastasis, invasion depth, tumour-lymph node metastasis (TNM) staging and tumour size (maximum diameter). This study also investigated the relations and possible mechanisms of the expression differences of ANXA1 protein in carcinogenesis, progression and prognosis of GC.

MATERIALS AND METHODS

Specimen collection and handling

Fifteen cases of GAC and paired gastric mucosa tissues were obtained from the First Affiliated Hospital of Xinjiang Medical University from June 2009 to October 2009 and used as the surgical resection specimens. Six female and nine male subjects aged 40-81 years (mean age of 56 years) and classified in TNM stages I to IV participated in this study. GC and paragastric mucosa tissues (located away from the primary tumour > 5 cm) with a size of approximately 1.0 cm^2 were obtained within 30 min of surgical resection. The tissues were washed immediately and repeatedly with normal saline to remove blood and other tissues. Afterwards, these tissues were stored at -80 ℃ in a refrigerator for future proteomics and western blot analysis. Informed consent was obtained from the patients to allow the collection and use of the samples. The procedures were also reviewed and approved by Xinjiang Medical Ethics Committee.

LCM for target cells and protein sample preparation

Frozen sections of GAC and paragastric mucosa tissues (8-10 μm) were prepared, affixed on LCM-specific film slides, fixed with 75% ethanol and stained with methyl green dye (Sigma-Aldrich, United States). The LCM system (Leica AS, Germany) was manipulated to determine GAC and NGEC. Tissue cell lysate was added and the total proteins of the purified GAC and NGEC were extracted. 2D Quant Kit (Amersham Biosciences, Sweden) was used to determine the protein concentration. We performed 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to pre-separate the total proteins of cleaved GAC and NGEC with a sample

loading volume of 100 μg. The gel was then stained with Coomassie R-250. The parallel gel bands were cut to obtain 36 pairs of protein gel bands. The obtained bands were washed, bleached, dehydrated, reduced with dithiothreitol (Amersham Biosciences, Sweden), alkylated with IAA and vacuum dried by centrifugation. Trypsin (Promega Corporation, United States) was added to initiate digestion. The peptides were extracted, mixed and dried. Afterwards, $^{18}O/^{16}O$ notation was performed by adding 8 μL of $H_2{}^{18}O$ (or $H_2{}^{16}O$; Huayi Isotope Corporation, Jiangsu) and 2 μL of acetonitrile (Sigma-Aldrich Inc., United States) to the mixed polypeptides, which also contained immobilized trypsin (Pierce, United States). The resulting mixture was then incubated at 37 ℃ for 24 h. At the end of the labelling reaction, 1 μL of formic acid was added to terminate the reaction.

Protein identification and mass data analysis

The mobile phase solution A (0.1% formic acid in water) was added into Eppendorf tubes containing different peptide mixtures, shaken and centrifuged. The supernatant was extracted and added into a tapered bottom vial in the Ultimate FAMOS LC system autosampler. Isolation was performed using nano-RPLC-MS/MS instrument, in which an auxiliary pump was used to load the samples with mobile phase solution A at a flow rate of 20 μL/min for 10 min. The samples were then loaded in a pre-column and desalted. The pre-column was switched and connected to a capillary analytical column for gradient elution using the solvent gradient as follows: solution B [water/acetonitrile (v/v) containing 0.1% formic acid], 5% B, 0-10 min; 5%-90% B, 55 min; 90% B, 5 min; and 90%-0% B, 10 min. After equilibrium was reached for 15 min, separation was performed. Nanoliter analytical column flow rate was approximately 250 nL/min; therefore, the eluent could be placed directly in the Electrospray ionization ion Q-TOF mass spectrometer (Micromass Corporation, United Kingdom) for analysis. The standard peptide Glu-Fibrino peptide B was used as an external calibration standard of the mass spectrometer. The mass data provided peaklist files in Masslynx 4.0 software of the local Mascot 2.0 IPI database to search the protein database and identify the proteins. Quantitative analysis was performed in Masslynx according to the following procedures: the MS spectra containing the peptides used for quantification were obtained from the total ion chromatograph to integrate and form the spectrum of quantitative analysis; $^{16}O/^{18}O$ ratio was then calculated according to Eq. (1)[7].

Detection of differential ANXA1 expression levels by Western blot

Fifteen pairs of microdissected and purified GAC and NGEC were added to the tissue lysate pre-cooled at 4 ℃, vortexed and cleared in an ice bath for 30 min. Afterwards, the samples were centrifuged at 12000 r/min and 4 ℃ for 30 min. The supernatant (the total cellular protein) was then transferred to a new tube. The protein concentration was determined using Bradford method and the total protein was determined by separation *via* 10% PAGE at 100 V for approximately 2 h (loading volume of 40 μg). The protein was electronically transferred to a Polyvinylidene difluoride membrane. Rabbit anti-human ANXA1 antibody (1/500) was added and incubated at 4 ℃ overnight. HRP-labeled goat anti-mouse secondary antibody (1/2000) incubated at room temperature for 2 h was also added. Enhanced chemiluminescence reagent lightening, developing and fixing were conducted. The obtained images were scanned to calculate the relative expression levels of the differential proteins in Quantity One software.

Immunohistochemical detection of the tissue microarray of ANXA1 expression

A total of 75 pairs of human GC tissue microarray (Shanghai Outdo Biotech Co., Ltd., China), including paired GC tissues and paraGC tissues were obtained from 50 males and 25 females aged 30-84 years (average age of 63.6 years). Among these subjects, 12 cases were in Phase Ⅰ, 25 cases in Phase Ⅱ, 32 cases in Phase Ⅲ and 4 cases in Phase Ⅳ stage cases (according to the TNM classification Standard, 7th edition, developed by the International Union Against Cancer in 2009). The clinical pathological data were complete: the cases showing tumours that invaded the submucosa, muscularis, serosa and serosa were 6, 13, 46 and 10, respectively. A total of 34 cases showed high amounts of moderately differentiated adenocarcinoma and 41 cases showed low amounts of undifferentiated adenocarcinoma. No lymph node metastasis was observed in 30 cases, but lymph node metastasis was present in 45 cases. Distant metastasis was absent in 69 cases, but 6 patients exhibited distant metastasis. According to SP method and the manufacturer's instructions, tissue microarrays were subjected to conventional dewaxing hydration and retrieved using citrate antigen. Afterwards, 3% H_2O_2-formaldehyde was used to block endogenous peroxidase. ANXA1 primary antibody (1/100) was added and incubated at 4 ℃ overnight. The biolabelled secondary antibody and streptavidin-peroxidase solution were added. Each sample was washed with PBS and incubated at room temperature. The sample was then stained with DAB staining, restained with haematoxylin and eosin, dehydrated with graded alcohol and mounted using neutral gum. The primary antibody was replaced with PBS as the negative control sample; the known positive reaction chip was used as the positive control sample. IHC staining score was based on Formowitz comprehensive scoring method[8] and determined according to the staining intensity and percentage of positive cells in each section. Staining intensity was scored as follows: no staining, 0; pale yellow, 1; brownish-yellow, 2; and tan, 3. At least 10 high-power fields (× 200) were randomly selected for each point and at least 1000 cells were counted. Among the total number of cells, the following percentages were obtained: 5% positively-stained cells scored as 0; 5%-25% scored as 1; 26%-50% scored as 2; 51%-75% scored as 3;

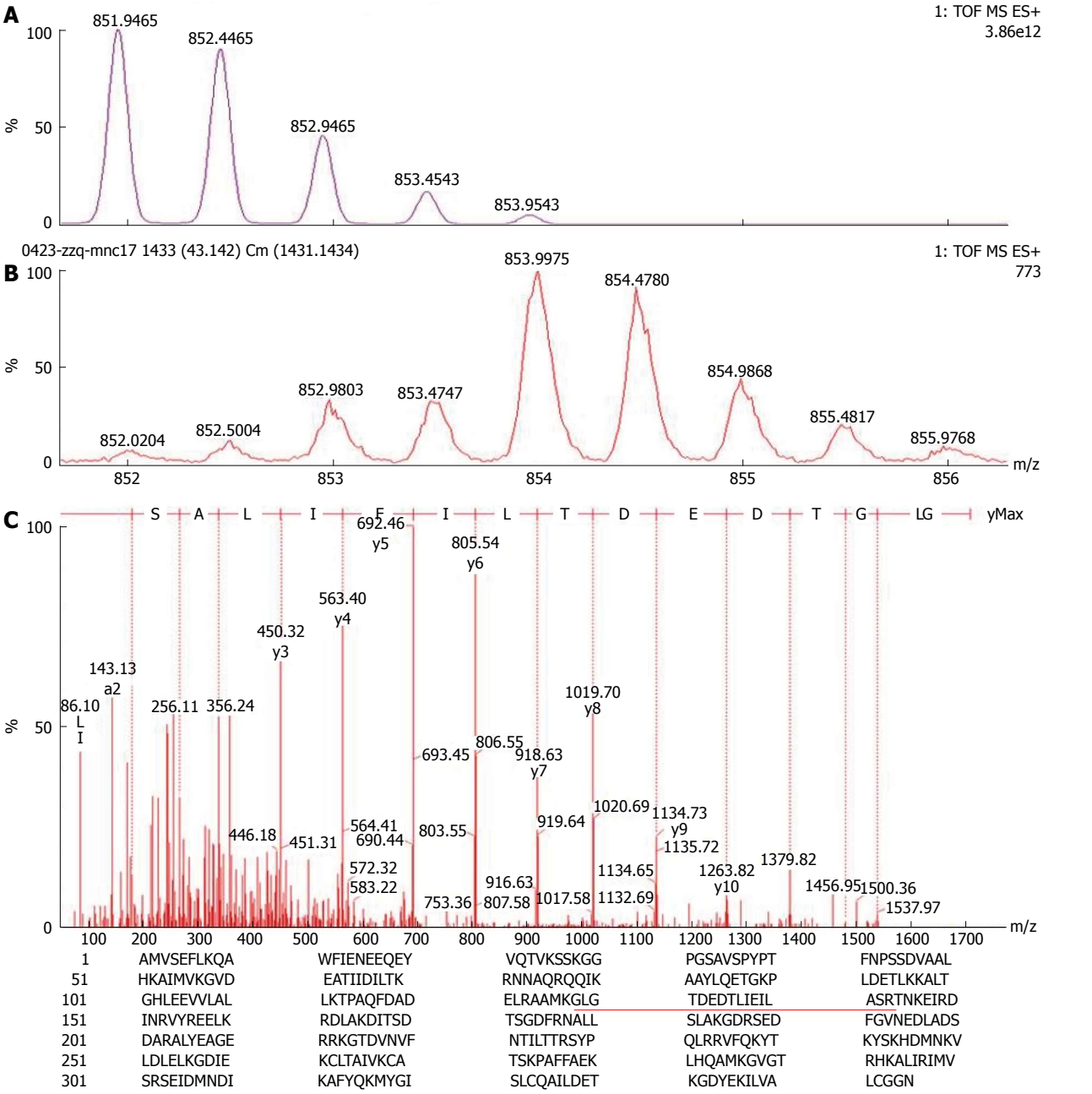


Figure 1 Mass spectrum of the isotopic distribution patterns of Annexin A1 (GLGTDEDTLIEILASR) and ESI-Q-TOF-MS analysis results. A: Theoretical isotopic distribution patterns (M0, relative abundance of theoretical monoisotopic peaks, M2, relative abundance of theoretical 2 Da monoisotopic peaks; M4, relative abundance of theoretical 4Da-monoisotopic peaks); B: Isotopic distribution patterns after ^{18}O labelling, I0, actual detected relative abundance of theoretical monoisotopic peaks,I2, actual detected relative abundance of theoretical 2Da-monoisotopic peaks; I4, actual detected relative abundance of theoretical 4Da-monoisotopic peaks; C: Amino acid sequence at *m/z* 854.00 was identified as GLGTDEDTLIEILASR; D: Protein sequence of Annexin A1 (ANXA1) showing the underlined MS/MS fragment matched the 128-143 amino acid residues of ANXA1.

> 75% scored as 4. The total score of staining intensity and the score of the percentage of positive cells were shown as follows: 2 as negative (-); 2-3 as weakly positive (+); 4-5 as moderately positive (++); and 6 to 7 as strongly positive (+++).

Statistical analysis

SPSS 15.0 statistical software was used to analyze the experimental results. The relationship between differential protein expression and the clinicopathological parameters of GC from different samples was determined by conducting Wilcoxon rank sum test. The relative protein expression levels were expressed as mean ± SD. *t* test was performed and $P < 0.05$ was considered statistically significant.

RESULTS

Isolation and identification of differentially expressed proteins of GAC and NGEC

The total proteins of highly homogeneous GAC and NGEC (> 90%) purified by LCM were determined to

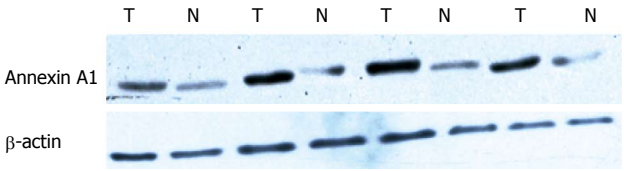


Figure 2 Expression of Annexin A1 protein in gastric adenocarcinoma cells and normal gastric epithelial cells by western blot analysis. T: Gastric adenocarcinoma cells; N: Normal gastric epithelial cells.

Table 1 Expression of annexin A1 protein in normal gastric epithelial cells and gastric adenocarcinoma cells

Group	*n*	Annexin A1 expression
NGEC	15	0.49 ± 0.082
GAC	15	1.06 ± 0.068[b]

[b]$P < 0.01$ *vs* NGEC. GAC: Gastric adenocarcinoma cells; NGEC: Normal gastric epithelial cells.

separate by 1D SDS-PAGE, gel digestion, extraction and ^{18}O labelling. These proteins were then identified by nano-RPLC-MS/MS. According to the standard differential proteins (where $2 < {}^{18}O/{}^{16}O$ ratio < 0.5), a total of 78 differential proteins were identified, in which the expressions of 42 proteins, including ANXA1, ANXA2, ANXA4, Protein S100-A9, and HSP 90-alpha2 were upregulated in GC. By contrast, the expressions of 36 proteins were downregulated in GC, including RKIP, ADP/ATP translocase 2 and L-lactate dehydrogenase B. These differentially expressed proteins function as metabolic enzymes, enzyme proteins, cytoskeletal proteins, and signal transduction proteins; other proteins also exhibit unknown functions, in which ANXA1 expression was 2.17 times higher in GAC than in NGEC (Figure 1).

Western blotting analysis identification of the differential expression of ANXA1

β-actin was used as the internal standard in western blot and the maximum gray value of the strip was set as 1. The other gray value was divided by the maximum gray value, and the obtained ratio corresponded to the relative protein expression level. The results showed that ANXA1 was upregulated at a higher extent in GAC than in NGEC ($P < 0.01$). The quantitative relationships calculated from the grayscale analysis were the same as the proteomic analysis results (Figure 2, Table 1).

ANXA1 protein expression in tissue microarray of GC and para-tissues

IHC was performed to detect 150 points of the tissue microarray in human GC. The results showed that ANXA1 protein was positively expressed mainly in the cytoplasm and the nuclei of GC tissues and paratissues (normal mucosa). Positive expression was also observed in the stroma. ANXA1 protein was highly expressed in GC. The negative, weakly positive, moderately positive and strongly positive expression rates in GC and para-tissues were: 30.7%, 14.7%, 34.7% and 20.0% *vs* 49.3%, 33.3%, 17.3% and 0%, respectively ($w = 4599.0$, $P < 0.01$; Table 2) The ANXA1 expression was significantly related to the invasion depth, lymph node metastasis, distant metastasis and TNM staging ($P < 0.01$). By contrast, ANXA1 expression was not significantly related to age, gender, histological grade and tumour size ($P > 0.05$; Figure 3, Table 3).

Table 2 Annexin A1 expression in normal gastric mucosa and gastric carcinoma by immunohistochemistry *n* (%)

Tissue type	*n*	Annexin A1 cases				W	*P* value
		-	+	+ +	+ + +		
NGEC	75	37 (49.3)	25 (33.3)	13 (17.3)	0 (0)	4599	0
GAC	75	23 (30.7)	11 (14.7)	26 (34.7)	15 (20.0)		

$P < 0.01$ *vs* normal gastric mucosa group (NGEC). GAC: Gastric adenocarcinoma cells.

DISCUSSION

The mortality rate of GC ranks second among the mortality rates of malignant tumours and approximately 75 million people die of stomach cancer worldwide yearly[9]. The five-year survival rate of GC is only 20%-30%, and the five-year survival rate of radical resection of early GC can reach 90%-95%[10]. Molecular markers are considered as one of the most sensitive and effective indicators of tumour diagnosis, recurrence, metastasis and prognosis prediction. Therefore, the molecular markers closely associated with the development of GC and the relative pathogenesis should be determined in the early diagnosis of GC to improve treatment outcomes. Proteomics technology has an important function in the identification of tumour-related protein species in cancer development as well as in the discovery of tumour molecular markers and therapeutic targets. The present study employed simple LCM to solve problems about tumour proteomics heterogeneity, in which the purified cells exhibiting > 90% homogeneity were rapidly obtained. Using the advanced ^{18}O stable isotope labelling/MS quantitative proteomics technique, we set the normal gastric mucosa and GC tissue of humans as the targets in this study to screen and identify the differentially expressed proteins. A total of 78 differentially expressed proteins were found and 42 of such proteins were highly expressed in GC cell compared with NGEC. By contrast, 36 differentially expressed proteins were found at lower concentrations. These results provided information about differentially expressed proteins and the mechanism of GC carcinogenesis, development and screening of molecular markers.

During the screening of the identified differential proteins, ANXA1 was significantly upregulated in GC. ANXA1 is involved in various physiological and pathological processes, such as cell signal transduction, cell proliferation, differentiation and apoptosis as well as inflammation and immune response. Previous studies found that ANXA1 is upregulated in breast cancer[11],

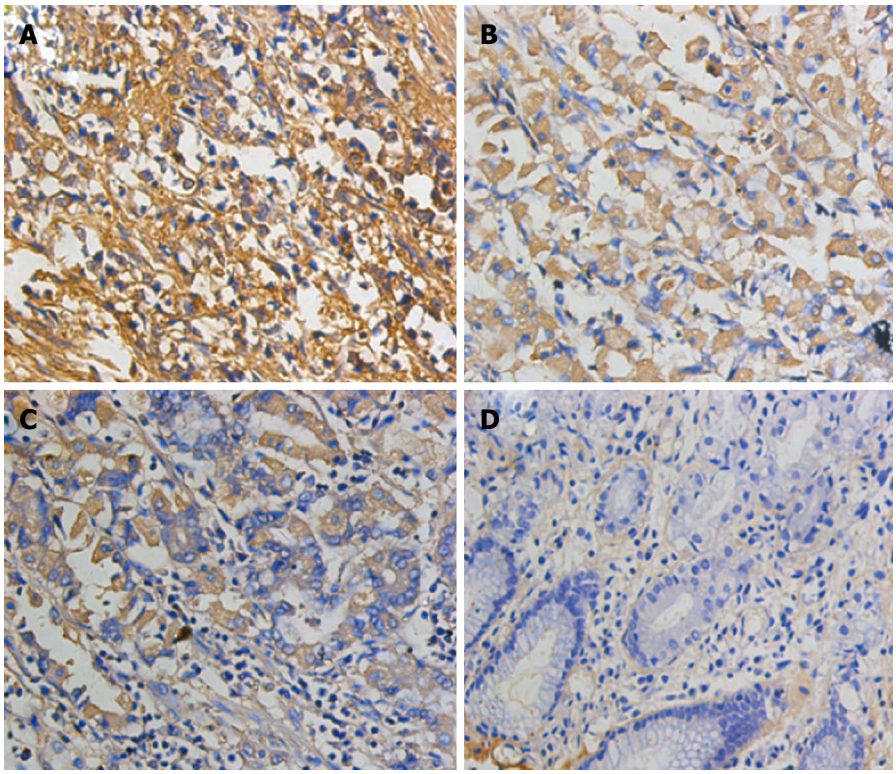


Figure 3 Representative immunohistochemical staining patterns of Annexin A1 expression in gastric carcinoma and normal gastric mucosa. A: Strongly positive staining; B: Moderately positive staining; C: Weakly positive staining; D: Negative staining (immunohistochemical staining × 400).

Table 3 Relationship between annexin A1 protein expression and clinicopathological parameters of gastric carcinoma *n* (%)

Parametes	*n*	ANXA1 cases				Wilcoxon	*P* value
		-	+	+ +	+ + +		
Sex						1039.5	0.290
Male	50	15 (30.0)	7 (14.0)	17 (34.0)	11 (22.0)		
Female	25	8 (32.0)	4 (16.0)	9 (36.0)	4 (16.0)		
Age (yr)						840.0	0.394
≤ 60	24	7 (29.2)	5 (20.8)	10 (41.7)	2 (8.3)		
> 60	51	16 (31.4)	6 (11.7)	16 (31.4)	13 (25.5)		
Histological differentiation						1350.0	0.520
High	34	10 (29.4)	4 (11.8)	12 (35.3)	8 (23.5)		
Moderate/poor	41	13 (31.7)	7 (17.1)	14 (34.1)	7 (17.1)		
Invasive depth						514.5	0.008
T1-2	19	10 (52.6)	4 (21.1)	3 (15.8)	2 (10.5)		
T3-4	56	13 (23.2)	7 (12.5)	23 (41.1)	13 (23.2)		
N stage						929.0	0.017
N0	30	12 (40.0)	6 (20.0)	10 (33.3)	2 (6.7)		
N1-3	45	11 (24.4)	5 (11.1)	16 (35.6)	13 (28.9)		
M stage						348.5	0.017
M0	69	23 (33.3)	10 (14.5)	25 (36.2)	11 (15.9)		
M1	6	0 (0)	1 (16.7)	1 (16.7)	4 (66.7)		
TNM stage						1014.5	0.000
Ⅰ-Ⅱ	37	20 (54.1)	4 (10.8)	11 (29.7)	2 (5.4)		
Ⅲ-Ⅳ	38	3 (2.6)	7 (10.5)	15 (42.1)	13 (44.7)		
Tumor size						1403.0	0.9735
≤ 5 cm	38	11 (28.9)	6 (15.8)	14 (36.8)	7 (18.4)		
< 5 cm	37	12 (32.4)	5 (13.5)	12 (32.4)	8 (21.6)		

ANXA1: Annexin A1; TNM: Tumour-lymph node metastasis.

lung cancer[12], pancreatic cancer[13], colorectal cancer[3,14] and bladder cancer[15]. By contrast, ANXA1 is downregulated in oral squamous cell carcinoma as well as nasopharyngeal, laryngeal and other head and neck cancer[16-18], oesophageal squamous cell carcinoma[2] and prostate cancer[19]. The dysfunction of ANXA1 is closely related to breast cancer, lung cancer, and pancreatic cancer as well as in other tumour invasion and metastasis. There-

fore, ANXA1 is considered as a risk factor affecting the survival of patients. Wang *et al*[20] used a immunohistochemical method and found that ANXA1 expression is 39% higher in the stomach/gastroesophageal junction adenocarcinoma and closely correlated with the pathological staging and distant metastasis of tumour, *i.e.*, higher clinical stages, particularly in distant lymph node metastasis, correspond to higher ANXA1 expression levels; higher tumour recurrence rates correspond to lower survival rate. This result suggested that the upregulation of ANXA1 could be used as a prognosis indicator in the stomach-oesophageal junction adenocarcinoma. Currently, the expression and function of ANXA1 in GC remain controversial.

In this study, quantitative proteomics was performed to screen ANXA1, revealing that ANXA1 was expressed 2.17 times higher in GC than in normal gastric mucosa. Western blotting and IHC of the tissue microarray also revealed the same results as proteomics analysis. In particular, the results showed that ANXA1 was significantly expressed at a higher extent in GC tissues than in paratissues; further analysis about the relations of ANXA1 and clinical parameters of human GAC revealed that ANXA1 expression was upregulated in tumour-penetrating serosa and thus invaded the paratissues. This result is different from human GAC normally confined to the mucosa, submucosa and muscularis; human GAC also possibly invaded the tumour in the lower serosa. ANXA1 was also highly expressed in human GAC with lymph node metastasis and distant metastasis compared with human GAC without lymph node metastasis and distant metastasis. As TNM staging increased, the positive expression rate of ANXA1 increased. However, no relationship was observed among ANXA1, patient's age, gender, histological grade and tumour size. This result suggested that ANXA1 was closely related to the biological behaviour of human GAC and involved in the development, invasion and metastasis of human GAC. Currently, the mechanism by which ANXA1 functions in the biological behaviour of GC remains unclear. Cheng *et al*[21] reported that ANXA1 can regulate GC invasion by mediating formyl peptide receptor (FPR)/extracellular signal-regulated kinase/integrin protein β-1 bind protein pathway; all of the three FPRs are involved in the regulation process. Kang *et al*[11] further found that *ANXA1* gene can decrease the activity and protein expression of matrix metalloproteinase-9 (MMP-9) transcriptional promoter by inhibiting the activity of nuclear factor κ-B. MMP-9 also has an important function in GC invasion and metastasis. Vascular endothelial growth factor (VEGF) is another important factor regulating angiogenesis, and angiogenesis is important in tumour growth and metastasis. Pin *et al*[22] found that VEGF-induced cell migration and angiogenesis of miR-196a can change the expression level of ANXA1, which is controlled by p38-ANXA1 signal conduction pathway. The function of *Helicobacter pylori* (*H. pylori*) infection in GC has also been demonstrated, showing that *H. pylori* can change the cellular ANXA1 localization[23]. This result suggests that ANXA1 may be involved in *H. pylori* infection-induced GC. Furthermore, these results can explain the possible mechanism by which ANXA1 participates in GAC biological behaviour to some extent.

A few reports about ANXA1 in GC have been published. For instance, Yapar *et al*[4] conducted an immunohistochemical study and found that ANXA1 expressed in GC is 56.3% higher than that in paratissues; this expression is also positively correlated with tumour invasion and lymph node metastasis; a high ANXA1 expression suggests poor prognosis of GC. Jorge *et al*[6] performed RT-PCR and immunohistochemistry, revealing that ANXA1 mRNA and protein expressions are increased in GC compared with normal gastric mucosa. The results of the present study are consistent with those in the aforementioned previous studies, although other studies have revealed contrasting results. In some studies, ANXA1 is downregulated in GC[4,24] and negatively correlated with tumour staging and lymph node metastasis[4]. The reasons may be described as follows: (1) different test conditions, antibodies, and test methods; and (2) different samples or different types and pathological staging parameters of GC samples. The intracellular ANXA1, which is mainly in the cytoplasm, is possibly redistributed at different stimulations. For example, ANXA1 likely enters the nucleus as induced by an epidermal growth factor (EGF) under oxidation conditions or heat shock; by contrast, ANXA1 is transferred to the membrane and then secreted out of the cells as stimulated by GC or phorbol-12-myristate-13-acetate[25,26]. Therefore, the upregulation and downregulation of ANXA1 in GC may be associated with different stages of GC pathology. Different levels of EGF and GC *in vivo* may possibly induce cellular ANXA1 relocalization. ANXA1 may also be involved in GC occurrence and development *via* different pathways and mechanisms.

In summary, the present study provided valuable information to clarify GC pathogenesis. This study also presented the basic foundation to screen cancer biomarkers. After the differential proteins of GC were initially screened, the results suggested that identification and function *in vivo* and *in vitro* of some important differential proteins require further studies. Given that ANXA1 was also expressed in gastric stromal cells, ANXA1 expression in GC may be difficult to assess by IHC. Nevertheless, the study on immunohistochemical tissue microarray revealed the relationship between ANXA1 and GC clinicopathological parameters. We found that the upregulation of ANXA1 in human GAC was closely related to the depth of tumour invasion, lymph node metastasis, distant metastasis and TNM stage. The results also suggested that ANXA1 was possibly involved in tumour invasion and metastasis. The high expression of ANXA1 suggested poor prognosis of GC. The mechanism by which ANXA1 participated in the GC biological behaviour should be further studied. With the continuous development of this research, ANXA1 may be used in early

cancer detection, diagnosis and treatment. ANXA1 may also become an indicator of cancer prognosis and a new target of cancer therapy, thereby providing new ideas of GC diagnosis and treatment.

COMMENTS

Background

Gastric cancer (GC) is a common digestive tract cancer because of the lack of early diagnosis strategies; a previous study revealed that GC cases are usually diagnosed when the disease is at an advanced stage.

Research frontiers

During the screening of the identified differential proteins, Annexin A1 (ANXA1) was significantly upregulated in GC. ANXA1 is involved in various physiological and pathological processes, such as cell signal transduction, cell proliferation, differentiation and apoptosis as well as inflammation and immune response. Previous studies found that ANXA1 is upregulated in breast cancer, lung cancer, pancreatic cancer, colorectal cancer and bladder cancer. By contrast, ANXA1 is downregulated in oral squamous cell carcinoma as well as nasopharyngeal, laryngeal and other head and neck cancer, oesophageal squamous cell carcinoma and prostate cancer.

Innovations and breakthroughs

The study performed laser capture microdissection to investigate the relation of ANXA1 expression in GC to the clinical parameters and to obtain purified gastric adenocarcinoma cells (GAC) and normal gastric epithelial cells (NGEC). $^{18}O/^{16}O$ was used to label the digested peptides in the mixture of GAC and NGEC. Nanoliter-reverse-phase liquid chromatography-mass/mass spectrometry was performed to identify and quantify the differentially expressed proteins.

Applications

ANXA1 may be used in early cancer detection, diagnosis and treatment. ANXA1 may also become an indicator of cancer prognosis and a new target of cancer therapy, thereby providing new ideas of GC diagnosis and treatment.

Peer review

This study is realistic significance to the GC, This manuscript is well written. The data is interesting and worthy for publication.

REFERENCES

1 **Wadhwa R**, Taketa T, Sudo K, Blum MA, Ajani JA. Modern oncological approaches to gastric adenocarcinoma. *Gastroenterol Clin North Am* 2013; **42**: 359-369 [PMID: 23639645 DOI: 10.1016/j.gtc.2013.01.011]

2 **Moghanibashi M**, Jazii FR, Soheili ZS, Zare M, Karkhane A, Parivar K, Mohamadynejad P. Proteomics of a new esophageal cancer cell line established from Persian patient. *Gene* 2012; **500**: 124-133 [PMID: 22441127 DOI: 10.1016/j.gene.2012.03.038]

3 **Roth U**, Razawi H, Hommer J, Engelmann K, Schwientek T, Müller S, Baldus SE, Patsos G, Corfield AP, Paraskeva C, Hanisch FG. Differential expression proteomics of human colorectal cancer based on a syngeneic cellular model for the progression of adenoma to carcinoma. *Proteomics* 2010; **10**: 194-202 [PMID: 19899082 DOI: 10.1002/pmic.200900614]

4 **Yapar EG**, Ekici E, Karasahin E, Gökmen O. Sonographic features of tuberculous peritonitis with female genital tract tuberculosis. *Ultrasound Obstet Gynecol* 1995; **6**: 121-125 [PMID: 8535914 DOI: 10.1007/s10585-008-9178-y]

5 **Sato Y**, Kumamoto K, Saito K, Okayama H, Hayase S, Kofunato Y, Miyamoto K, Nakamura I, Ohki S, Koyama Y, Takenoshita S.. Up-regulated Annexin A1 expression in gastrointestinal cancer is associated with cancer invasion and lymph node metastasis. *Exp Ther Med* 2011; **2**: 239-243 [PMID: 22977491]

6 **Jorge YC**, Mataruco MM, Araújo LP, Rossi AF, de Oliveira JG, Valsechi MC, Caetano A, Miyazaki K, Fazzio CS, Thomé JA, Rahal P, Oliani SM, Silva AE. Expression of annexin-A1 and galectin-1 anti-inflammatory proteins and mRNA in chronic gastritis and gastric cancer. *Mediators Inflamm* 2013; **2013**: 152860 [PMID: 23431236 DOI: 10.1155/2013/152860]

7 **Qian WJ**, Monroe ME, Liu T, Jacobs JM, Anderson GA, Shen Y, Moore RJ, Anderson DJ, Zhang R, Calvano SE, Lowry SF, Xiao W, Moldawer LL, Davis RW, Tompkins RG, Camp DG, Smith RD. Quantitative proteome analysis of human plasma following in vivo lipopolysaccharide administration using 16O/18O labeling and the accurate mass and time tag approach. *Mol Cell Proteomics* 2005; **4**: 700-709 [PMID: 15753121]

8 **Fromowitz FB**, Viola MV, Chao S, Oravez S, Mishriki Y, Finkel G, Grimson R, Lundy J. ras p21 expression in the progression of breast cancer. *Hum Pathol* 1987; **18**: 1268-1275 [PMID: 3315956]

9 **Murray CJ**, Lopez AD. Mortality by cause for eight regions of the world: Global Burden of Disease Study. *Lancet* 1997; **349**: 1269-1276 [PMID: 9142060]

10 **Peddanna N**, Holt S, Verma RS. Genetics of gastric cancer. *Anticancer Res* 1995; **15**: 2055-2064 [PMID: 8572602]

11 **Kang H**, Ko J, Jang SW. The role of annexin A1 in expression of matrix metalloproteinase-9 and invasion of breast cancer cells. *Biochem Biophys Res Commun* 2012; **423**: 188-194 [PMID: 22640735 DOI: 10.1016/j.bbrc.2012.05.114]

12 **Biaoxue R**, Xiling J, Shuanying Y, Wei Z, Xiguang C, Jinsui W, Min Z. Upregulation of Hsp90-beta and annexin A1 correlates with poor survival and lymphatic metastasis in lung cancer patients. *J Exp Clin Cancer Res* 2012; **31**: 70 [PMID: 22929401]

13 **Chen CY**, Shen JQ, Wang F, Wan R, Wang XP. Prognostic significance of annexin A1 expression in pancreatic ductal adenocarcinoma. *Asian Pac J Cancer Prev* 2012; **13**: 4707-4712 [PMID: 23167407]

14 **Duncan R**, Carpenter B, Main LC, Telfer C, Murray GI. Characterisation and protein expression profiling of annexins in colorectal cancer. *Br J Cancer* 2008; **98**: 426-433 [PMID: 18071363]

15 **Li CF**, Shen KH, Huang LC, Huang HY, Wang YH, Wu TF. Annexin-I overexpression is associated with tumour progression and independently predicts inferior disease-specific and metastasis-free survival in urinary bladder urothelial carcinoma. *Pathology* 2010; **42**: 43-49 [PMID: 20025479 DOI: 10.3109/00313020903434405]

16 **Faria PC**, Sena AA, Nascimento R, Carvalho WJ, Loyola AM, Silva SJ, Durighetto AF, Oliveira AD, Oliani SM, Goulart LR. Expression of annexin A1 mRNA in peripheral blood from oral squamous cell carcinoma patients. *Oral Oncol* 2010; **46**: 25-30 [PMID: 19833546 DOI: 10.1016/j.oraloncology.2009.09.003]

17 **Alves VA**, Nonogaki S, Cury PM, Wünsch-Filho V, de Carvalho MB, Michaluart-Júnior P, Moyses RA, Curioni OA, Figueiredo DL, Scapulatempo-Neto C, Parra ER, Polachini GM, Silistino-Souza R, Oliani SM, Silva-Júnior WA, Nobrega FG, Tajara EH, Zago MA. Annexin A1 subcellular expression in laryngeal squamous cell carcinoma. *Histopathology* 2008; **53**: 715-727 [PMID: 19076685 DOI: 10.1111/j.1365-2559.2008.03186.x]

18 **Cheng AL**, Huang WG, Chen ZC, Peng F, Zhang PF, Li MY, Li F, Li JL, Li C, Yi H, Yi B, Xiao ZQ. Identification of novel nasopharyngeal carcinoma biomarkers by laser capture microdissection and proteomic analysis. *Clin Cancer Res* 2008; **14**: 435-445 [PMID: 18223218 DOI: 10.1158/1078-0432.CCR-07-1215]

19 **Schaeffer EM**, Marchionni L, Huang Z, Simons B, Blackman A, Yu W, Parmigiani G, Berman DM. Androgen-induced programs for prostate epithelial growth and invasion arise in embryogenesis and are reactivated in cancer. *Oncogene* 2008; **27**: 7180-7191 [PMID: 18794802 DOI: 10.1038/onc.2008.327]

20 **Wang KL**, Wu TT, Resetkova E, Wang H, Correa AM, Hofstetter WL, Swisher SG, Ajani JA, Rashid A, Hamilton SR, Albarracin CT. Expression of annexin A1 in esophageal and esophagogastric junction adenocarcinomas: association with poor outcome. *Clin Cancer Res* 2006; **12**: 4598-4604 [PMID:

16899607]
21 **Cheng TY**, Wu MS, Lin JT, Lin MT, Shun CT, Huang HY, Hua KT, Kuo ML. Annexin A1 is associated with gastric cancer survival and promotes gastric cancer cell invasiveness through the formyl peptide receptor/extracellular signal-regulated kinase/integrin beta-1-binding protein 1 pathway. *Cancer* 2012; **118**: 5757-5767 [PMID: 22736399 DOI: 10.1002/cncr.27565]
22 **Pin AL**, Houle F, Fournier P, Guillonneau M, Paquet ÉR, Simard MJ, Royal I, Huot J. Annexin-1-mediated endothelial cell migration and angiogenesis are regulated by vascular endothelial growth factor (VEGF)-induced inhibition of miR-196a expression. *J Biol Chem* 2012; **287**: 30541-30551 [PMID: 22773844 DOI: 10.1074/jbc.M112.393561]
23 **Lin LL**, Huang HC, Ogihara S, Wang JT, Wu MC, McNeil PL, Chen CN, Juan HF. Helicobacter pylori Disrupts Host Cell Membranes, Initiating a Repair Response and Cell Proliferation. *Int J Mol Sci* 2012; **13**: 10176-10192 [PMID: 22949854 DOI: 10.3390/ijms13]
24 **Zhu F**, Xu C, Jiang Z, Jin M, Wang L, Zeng S, Teng L, Cao J. Nuclear localization of annexin A1 correlates with advanced disease and peritoneal dissemination in patients with gastric carcinoma. *Anat Rec* (Hoboken) 2010; **293**: 1310-1314 [PMID: 20665809 DOI: 10.1002/ar.21176]
25 **Solito E**, de Coupade C, Parente L, Flower RJ, Russo-Marie F. IL-6 stimulates annexin 1 expression and translocation and suggests a new biological role as class II acute phase protein. *Cytokine* 1998; **10**: 514-521 [PMID: 9702415]
26 **Rhee HJ**, Kim GY, Huh JW, Kim SW, Na DS. Annexin I is a stress protein induced by heat, oxidative stress and a sulfhydryl-reactive agent. *Eur J Biochem* 2000; **267**: 3220-3225 [PMID: 10824106]

P- Reviewers: Okada H, Thuss-Patience PC **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.v19.i43.7804

World J Gastroenterol 2013 November 21; 19(43): 7804-7812
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

META-ANALYSIS

Is Dor fundoplication optimum after laparoscopic Heller myotomy for achalasia? A meta-analysis

Ming-Tian Wei, Ya-Zhou He, Xiang-Bing Deng, Yuan-Chuan Zhang, Ting-Han Yang, Cheng-Wu Jin, Bing Hu, Zi-Qiang Wang

Ming-Tian Wei, Ya-Zhou He, Xiang-Bing Deng, Yuan-Chuan Zhang, Ting-Han Yang, Cheng-Wu Jin, Zi-Qiang Wang, Department of Gastrointestinal Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Bing Hu, Endoscopic Center, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Author contributions: Wang ZQ and Wei MT designed the research; Wang ZQ and Hu B provided supervision; Wei MT, He YZ and Deng XB performed the research; Zhang YC, Yang TH and Jin CW performed a literature search and collected the data; Wei MT and He YZ analyzed the data and wrote the paper.
Supported by National Natural Science Foundation of China, Grant 81172373
Correspondence to: Zi-Qiang Wang, Professor, Department of Gastrointestinal Surgery, West China Hospital, Sichuan University, No. 37 Guo Xue Alley, Chengdu 610041, Sichuan Province, China. wangzqzyh@163.com
Telephone: +86-28-85422480 Fax: +86-28-81654035
Received: July 14, 2013 Revised: August 24, 2013
Accepted: September 16, 2013
Published online: November 21, 2013

Abstract

AIM: To compare the outcome of acid reflux prevention by Dor fundoplication after laparoscopic Heller myotomy (LHM) for achalasia.

METHODS: Electronic database PubMed, Ovid (Evidence-Based Medicine Reviews, EmBase and Ovid MEDLINE) and Cochrane Library were searched between January 1995 and September 2012. Bibliographic citation management software (EndNote X3) was used for extracted literature management. Quality assessment of random controlled studies (RCTs) and non-RCTs was performed according to the Cochrane Handbook for Systematic Reviews of Interventions 5.1.0 and a modification of the Newcastle-Ottawa Scale, respectively. The data were analyzed using Review Manager (Version 5.1), and sensitivity analysis was performed by sequentially omitting each study.

RESULTS: Finally, 6 studies, including a total of 523 achalasia patients, compared Dor fundoplication with other types of fundoplication after LHM (Dor-other group), and 8 studies, including a total of 528 achalasia patients, compared Dor fundoplication with no fundoplication after LHM (Dor-no group). Dor fundoplication was associated with a significantly higher recurrence rate of clinical regurgitation and pathological acid reflux compared with the other fundoplication group (OR = 7.16, 95%CI: 1.25-40.93, P = 0.03, and OR = 3.79, 95%CI: 1.23-11.72, P = 0.02, respectively). In addition, there were no significant differences between Dor fundoplication and no fundoplication in all subjects. Other outcomes, including complications, dysphagia, postoperative physiologic testing, and operation-related data displayed no significant differences in the two comparison groups.

CONCLUSION: Dor fundoplication is not the optimum procedure after LHM for achalasia. We suggest more attention should be paid on quality of life among different fundoplications.

Key words: Laparoscopic Heller myotomy; Dor fundoplication; Gastroesophageal reflux; Achalasia; Meta-analysis

Core tip: Laparoscopic Heller myotomy (LHM) is commonly used to treat achalasia and an antireflux procedure is added after LHM for prevention of gastroesophageal reflux (GER). However, there is no consensus on whether Dor fundoplication is the optimum procedure after LHM for the prevention of GER. We conducted this meta-analysis to assess Dor fundoplication com-

pared with non-fundoplication surgery or other types of fundoplication surgery for achalasia. The results indicated higher recurrence rate of clinical regurgitation and pathological acid reflux in Dor fundoplication indicating that Dor fundoplication is not the optimum procedure for the prevention of GER after LHM in achalasia patients.

Wei MT, He YZ, Deng XB, Zhang YC, Yang TH, Jin CW, Hu B, Wang ZQ. Is Dor fundoplication optimum after laparoscopic Heller myotomy for achalasia? A meta-analysis. *World J Gastroenterol* 2013; 19(43): 7804-7812 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7804.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7804

INTRODUCTION

Achalasia has generally been accepted as an autoimmune esophageal motility disorder resulting from the loss of inhibitory nerve endings in the myenteric plexus of the esophagus[1]. Pathophysiologically characterized by poor relaxation of lower esophageal sphincter (LES) and aperistalsis of the esophageal body, achalasia presents mainly relevant symptoms such as dysphagia, regurgitation, heartburn, and chest pain. The commonly used treatment of achalasia involves medicine therapy, endoscopic pneumatic dilation, and surgical myotomy with the aim of eliminating the high LES pressure. Previous studies have reported better long-term satisfaction with surgical myotomy than with drug medicine therapy or pneumatic dilation[2-4]. Kostic and colleagues in 2007 also demonstrated the superiority of laparoscopic Heller myotomy (LHM) to pneumatic dilation for achalasia patients[5]. As a result, LHM is routinely considered an option for achalasia patients.

However, although LHM has been previously demonstrated to have positive long-term outcomes for achalasia patients, gastroesophageal reflux (GER) after LHM is commonly regarded as one of the main failures of surgical treatment. For this reason, many surgeons suggest the addition of a fundoplication to LHM for the prevention of acid reflux, and anterior 180° Dor fundoplication is currently well recognized as the best choice[6]. Recently, in 2012, a review conducted by Mayo reconfirmed the efficacy of anti-acid reflux fundoplication following LHM both on pH monitoring and symptom relief; however, the clinical differences between Dor fundoplication and posterior 270° Toupet fundoplication have not been verified. In addition, the Mayo review provides limited evidence without pooling available data from the included studies[7]. Thus, it also remains controversial that Dor fundoplication is the optimum procedure for the prevention of postoperative GER after LHM in achalasia patients.

To address these issues, our team conducted the following meta-analysis to compare Dor fundoplication plus LHM with LHM alone (Dor-no group) and LHM plus other types of fundoplication (Dor-other group), namely, 270° Toupet and 360° Nissen fundoplication. The assessed outcomes included: (1) the primary endpoints of postoperative GER, dysphagia, and perforation; and (2) the secondary endpoints of other symptoms, quality of life, operation-related data, complications, and postoperative physiologic testing.

MATERIALS AND METHODS

This meta-analysis was conducted following the Cochrane Handbook for Systematic Reviews of Interventions 5.1.0 (updated March 2011) to ensure data quality (http://www.cochrane.org/training/cochrane-handbook).

Search for studies

Electronic databases PubMed, Ovid (EBM Reviews, EmBase and Ovid MEDLINE) and Cochrane Library were searched. Moreover, previously published reviews on the topic of interest were obtained and checked. We traced the reference list of relevant articles and used Google Scholar to find potential studies. The search terms were as follows: combined terms of "fundoplication" and "achalasia" using [Mesh] or [Keyword]. The electronic search was up to September 2012 from January 1995 with no limitation on language.

Study selection

Study designs included random controlled studies (RCTs), clinical controlled studies, cohort studies, case-control studies, and case series.

The inclusion criteria were as follows: (1) diagnosis of achalasia confirmed in an adult patient; (2) the surgical procedure compares Dor fundoplication with other fundoplication types (none, Toupet and Nissen); (3) laparoscopic Heller myotomy; and (4) available data for each comparison. We excluded: studies including (1) achalasia in children and pregnancy; (2) one type of fundoplication; (3) special surgical procedure such as anterior 120° wrap or Watson wrap[8]; and (4) studies lacking available data.

We imported the search results into bibliographic citation management software (EndNote X3). Two reviewers independently screened studies by reading titles and abstracts to roughly identify potential reports. The full texts of articles for all references identified as matching the inclusion criteria were obtained. Inclusion criteria were applied to the full texts. Disagreement was resolved through discussion and asking for advice from corresponding authors. The flow chart of study selection was made following the PRISMA statement (http://prisma-statement.org/statement.htm).

Data extraction and quality assessment

Two reviewers independently extracted data from eligible studies, and any disagreement was adjudicated by discussion or consulting the corresponding author. Base-

Table 1 Checklist of quality assessment and scoring of non-random controlled studies

Checklist
Selection
Is the subject definition adequate or described? (if yes, one star)
Were the subjects representative of the total population? (one star, if truly or obviously; no stars if subjects were selected group or not described)
Comparability
Did the study have no differences between Dor fundoplication and no fundoplication or other types of fundoplication? Major factors for consideration were age, gender, symptoms, preoperative therapy (pneumatic dilation and botulin toxin injection), and preoperative diagnostic test (endoscopy parameter and barium swallow) (if yes, two stars; one star if there were no other differences between the two groups even if one or more of these five characteristics was not reported; no star was assigned if the two groups differed)
Outcome assessment
Clearly defined outcome of interest (if yes, one star)
Adequacy of follow-up (one star if less than 20% of achalasia patients lost to follow-up, otherwise no stars)

line information included first author, published year, fundoplication type, study design, region, numbers of cases, and mean age among other parameters. Furthermore, the following outcome data were extracted: (1) the primary outcomes of GER-related clinical regurgitation and pathological acid reflux, dysphagia, and perforation; and (2) the secondary endpoints included other symptoms, quality of life, operation-related data (operation time and hospital stay time), complications, postoperative physiologic testing (LES pressure, DeMeester score and percent total time pH $\leqslant$ 4).

Quality assessment of RCTs was performed by two reviewers according to the Cochrane Handbook for Systematic Reviews of Interventions 5.1.0 based on the following aspects: random sequence generation, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other sources of bias. Three bias levels including low risk, high risk and unclear were assigned to every study aspect. Studies with more "low risk" bias assignations were recognized as superior. For non-random controlled studies, a modification of the Newcastle-Ottawa Scale (NOS)[9,10] was used as an assessment tool for selection, comparability and outcome assessment. Out of a total of six scores, studies valued more than four stars were recognized as being moderate to high quality. The detailed checklist is shown in Table 1.

Statistical analysis

The data were analyzed using Review Manager (Version 5.1). OR or RD and MD were used for analyzing dichotomous data and continuous data, respectively. Heterogeneity was measured with the I^2 index and P value. A random effect model was used when $I^2 > 50\%$. Otherwise, a fixed-effect model was considered. SD was estimated by a formula when only a range was reported: Estimate SD = Range/4 ($15 < n < 70$); Range/6 ($n > 70$)[11]. The value of $P < 0.05$ was considered to indicate statistical significance. Sensitivity analysis was performed by sequentially omitting each study.

RESULTS

Characteristics of pooled studies

A total of 731 potential abstracts were identified in the primary search of the electronic databases. A flow diagram of the detailed selection process is shown in Figure 1. Finally, 6 studies (2 RCTs and 4 non-RCTs), including a total of 523 achalasia patients, compared Dor fundoplication with other types of fundoplication (Toupet and Nissen fundoplication) after LHM, and 8 studies (3 RCTs and 5 non-RCTs), including a total of 528 achalasia patients, compared Dor fundoplication with no fundoplication after LHM[12-24]. In the 5 RCTs, two reported the same population group but differed in the main outcomes. Thus, we just extracted useful data integrated from both articles[19,23]. In addition, in the 8 non-RCTs, two studies were conducted by the same research group, who reported on the achalasia population with short- and long-term outcomes, and we chose the latter for our meta-analysis[15,24]. In one non-RCT, we divided the pooled data into two comparisons from the three reported subgroups[20]. In terms of non-Dor fundoplication, surgical fundoplication included 2 studies that used Nissen fundoplication, 4 studies with Toupet fundoplication and no fundoplication was used in the other studies. Table 2 offers the baseline characteristics of all studies.

Quality judgments of studies

In the pooled studies, 5 were RCTs, and 8 were non-RCTs. We used two methods to assess the quality of RCTs and non-RCTs, respectively. Table 3 lists the quality of RCTs according to the Cochrane Handbook for Systematic Reviews of Interventions 5.1.0. All the studies described the random sequence generation method used. Two studies generated sequence using a permuted block size of 4. Two used computer-generated random numbers, and 1 used a random number table generated in Microsoft Excel. In regards to allocation concealment, 3 studies used sealed opaque envelopes, 1 used Random Allocation Software version 1.0, and 1 study was unclear about the allocation concealment method used. In term of blinding, double blinding is difficult, and risks were judged by whether the outcome was likely influenced by the lack of blinding. In the 5 RCTs, only two trials reported double blinding of all recruited patients and researchers involved in the evaluation. Concerning selective reporting, although the protocol of each study was unavailable, the published outcomes included all the outcomes detailed in the method. Other sources of bias

Table 2 Basic characteristics of all pooled studies in the meta-analysis (Dor-other/no group)

Ref.	Patients (*n*)		Follow-up (mean ± SD or range)		Age (mean ± SD or range)	Type of fundoplication in control group	Study design	Country
	Dor group	Control group	Dor group	Control group				
Di Martino *et al*[13], 2011	30	26	24	24	42.8 ± 14.7	Other[1]	Prospective	Italy
Oelschlager *et al*[15], 2003	52	58	46 (1-85)	16 (1-38)	42.6 ± 15.5	Other[2]	Retrospective	United States
Rawlings *et al*[17], 2012	36	24	12	12	48.8 ± 13.0	Other[2]	RCT	United States
Rebecchi *et al*[18], 2008	72	72	125 (60-168)	125 (60-168)	49 (11-80)	Other[1]	RCT	Italy
Richardson *et al*[20], 2006	18	20	37 (2-97)	37 (2-97)	69 (15-80)	Other[2]	Retrospective	United States
Wright *et al*[24], 2007	52	63	46 ± 24	45 ± 17	42.5 (15.4)	Other[2]	Retrospective	United States
Dempsey *et al*[12], 2004	22	29	39 ± 22	26 ± 19	47.5 (12.6)	No	Retrospective	United States
Finley *et al*[14], 2007	71	24	6.9 ± 3.5	6.9 ± 3.5	47.9 (16-84)	No	Retrospective	Canada
Ramacciato *et al*[16], 2005	17	15	12	12	42.0 (14-77)	No	Retrospective	Italy
Richards *et al*[19], 2004	22	21	6	6	50 ± 12.7	No	RCT	United States
Richardson *et al*[20], 2006	18	14	37 (2-97)	37 (2-97)	69 (15-80)	No	Retrospective	United States
Simić *et al*[21], 2010	36	22	36	36	49.6 ± 29.2	No	RCT	Serbia
Tapper *et al*[22], 2008	75	99	8.4 ± 12.0	48.7 ± 34.6	47.0 ± 16.8	No	Prospective	United States
Torquati *et al*[23], 2006	22	21	NA	NA	50 ± 12.7	No	RCT	United States

[1]Other: Nissen fundoplication; [2]Other: Toupet fundoplication. NA: Not available; RCT: Random controlled trail.

Table 3 Quality assessment of random controlled studies in the meta-analysis based on the Cochrane Handbook version 5.1.0

Ref.	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other sources of bias
Rawlings *et al*[17], 2012	Low	Unclear	High	Low	Low	Low	Unclear
Rebecchi *et al*[18], 2008	Low	Low	High	Low	Low	Low	Unclear
Richards *et al*[19], 2004	Low	Low	Low	Low	Low	Low	Unclear
Simić *et al*[21], 2010	Low	Low	High	High	Unclear	low	Unclear
Torquati *et al*[23], 2006	Low	Low	Low	Low	Low	Low	Unclear

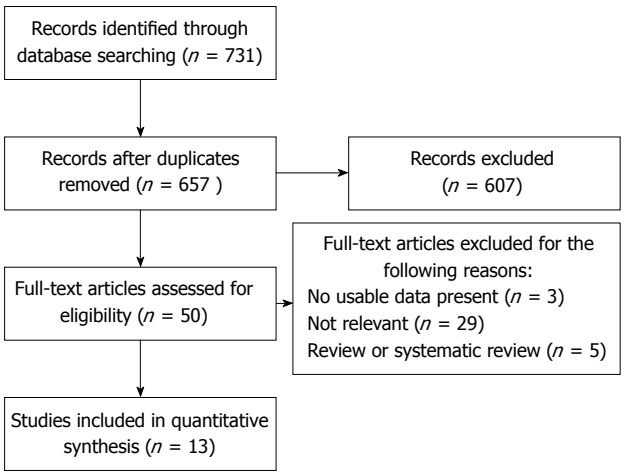


Figure 1 Flow diagram of meta-analysis study selection process.

were unclear in the included RCTs.

In term of the 8 non-RCTs, Table 4 lists the evaluation stars of each study followed by the modified NOS. In the selection of patients, one study included patients without continuity, which could hardly represent the total population[22]. Three studies (two studies reported the same patients group) received no stars in the domain of adequacy of follow-up, with a follow-up of 63.7%[20] and 30% (postoperative manometer), respectively[15,24]. Overall, all studies were evaluated as being moderate to high quality.

Outcomes in the Dor-other group

Primary endpoints: With respect to clinical regurgitation, 3 non-RCTs reported the available number of achalasia patients[13,18,20], and a fixed-effect model was used in the subgroup meta-analysis. Dor fundoplication was appraised to have a significantly higher recurrence rate of clinical regurgitation compared with other types of fundoplication (OR = 7.16, 95%CI: 1.25-40.93, P = 0.03 and heterogeneity I^2 = 0%) (Figure 2A). One study, which did not have an available number of achalasia patients, reported no significant difference in regurgitation frequency score (P = 0.546)[24].

In the pathological acid reflux analysis, 1 RCT and 2 non-RCTs were pooled, and a fixed-effect model was used[13,17,18]. The odds ratio was 3.79 in the Dor fundoplication group compared with the other fundoplication group (95%CI: 1.23-11.72, P = 0.02 and heterogeneity I^2 = 0%).

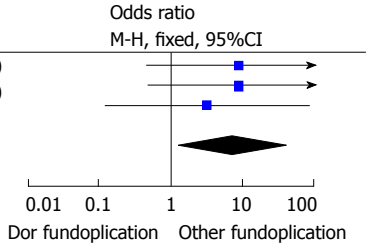
Perforation was estimated in 2 RCTs and 1 non-RCT, and a fixed-effect model was used. The subgroup analysis indicated no significant differences in the Dor-other group (RD = -0.00, 95%CI: -0.04-0.04, P = 0.94, heterogeneity I^2 = 0%) (Figure 2A).

Considering dysphagia, no significant symptom relief benefit was found for Dor fundoplication compared with other types of fundoplication, and a random-effect model was used (OR = 1.19, 95%CI: 0.16-8.67, P = 0.86 and heterogeneity I^2 = 77%) (Figure 2B). One study that lacked information on the number of acha-

A Clinical regurgitation

Study or subgroup	Dor fundoplication Events	Total	Other fundoplication Events	Total	Weight	Odds ratio M-H, fixed, 95%CI
Di Martino *et al*, 2011	4	30	0	26	33.1%	9.00 (0.46, 175.59)
Rebecchi *et al*, 2008	4	71	0	67	34.9%	9.00 (0.48, 170.44)
Richardson *et al*, 2006	1	12	0	12	32.0%	3.26 (0.12, 88.35)
Total (95%CI)		113		105	100.0%	7.16 (1.25, 40.93)
Total events	9		0			

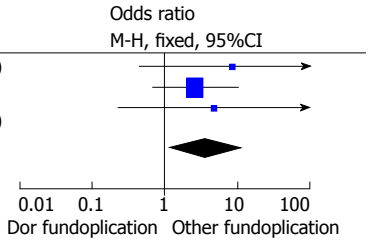
Heterogeneity: χ^2 = 0.26, *df* = 2 (*P* = 0.88); I^2 = 0%
Test for overall effect: Z = 2.21 (*P* = 0.03)



Pathological acid reflux

Study or subgroup	Dor fundoplication Events	Total	Other fundoplication Events	Total	Weight	Odds ratio M-H, fixed, 95%CI
Di Martino *et al*, 2011	4	30	0	26	12.8%	9.00 (0.46, 175.59)
Rawlings *et al*, 2012	10	24	4	19	73.2%	2.68 (0.68, 10.53)
Rebecchi *et al*, 2008	2	71	0	67	14.0%	4.86 (0.23, 103.03)
Total (95%CI)		125		112	100.0%	3.79 (1.23, 11.72)
Total events	16		4			

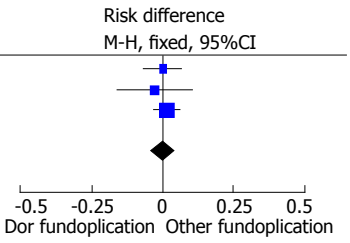
Heterogeneity: χ^2 = 0.60, *df* = 2 (*P* = 0.74); I^2 = 0%
Test for overall effect: Z = 2.32 (*P* = 0.02)



Perforation

Study or subgroup	Dor fundoplication Events	Total	Other fundoplication Events	Total	Weight	Odds ratio M-H, fixed, 95%CI
Di Martino *et al*, 2011	0	30	0	26	21.7%	0.00 (-0.07, 0.07)
Rawlings *et al*, 2012	2	36	2	24	22.4%	-0.03 (-0.16, 0.11)
Rebecchi *et al*, 2008	2	72	1	72	56.0%	0.01 (-0.03, 0.06)
Total (95%CI)		138		122	100.0%	0.00 (-0.04, 0.04)
Total events	4		3			

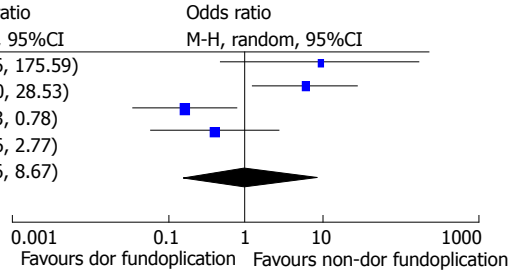
Heterogeneity: χ^2 = 0.46, *df* = 2 (*P* = 0.80); I^2 = 0%
Test for overall effect: Z = 0.07 (*P* = 0.94)



B Other fundoplication

Study or subgroup	Dor fundoplication Events	Total	Other fundoplication Events	Total	Weight	Odds ratio M-H, fixed, 95%CI
Di Martino *et al*, 2011	4	30	0	26	19.1%	9.00 (0.46, 175.59)
Oelschlager *et al*, 2003	9	52	2	58	27.6%	5.86 (1.20, 28.53)
Rebecchi *et al*, 2008	2	71	10	67	27.8%	0.17 (0.03, 0.78)
Richardson *et al*, 2006	2	12	4	12	25.4%	0.40 (0.06, 2.77)
Total (95%CI)		165		163	100.0%	1.29 (0.16, 8.67)
Total events	17		16			

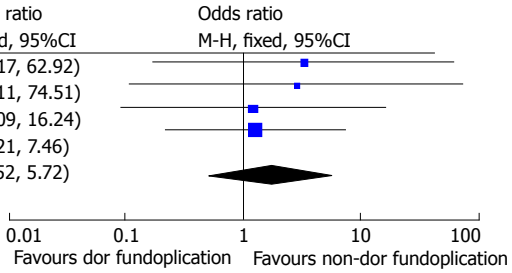
Heterogeneity: Tau^2 = 3.06; χ^2 = 12.91, *df* = 3 (*P* = 0.005); I^2 = 77%
Test for overall effect: Z = 0.17 (*P* = 0.86)



No fundoplication

Study or subgroup	Dor fundoplication Events	Total	Other fundoplication Events	Total	Weight	Odds ratio M-H, fixed, 95%CI
Finley *et al*, 2007	4	71	0	24	15.7%	3.27 (0.17, 62.92)
Ramacciato *et al*, 2005	1	17	0	15	10.9%	2.82 (0.11, 74.51)
Richardson *et al*, 2006-2	2	12	1	7	23.7%	1.20 (0.09, 16.24)
Simić *et al*, 2010	4	36	2	22	49.7%	1.25 (0.21, 7.46)
Total (95%CI)		136		68	100.0%	1.73 (0.52, 5.72)
Total events	11		3			

Heterogeneity: χ^2 = 0.46, *df* = 3 (*P* = 0.93); I^2 = 0%
Test for overall effect: Z = 0.89 (*P* = 0.37)



C

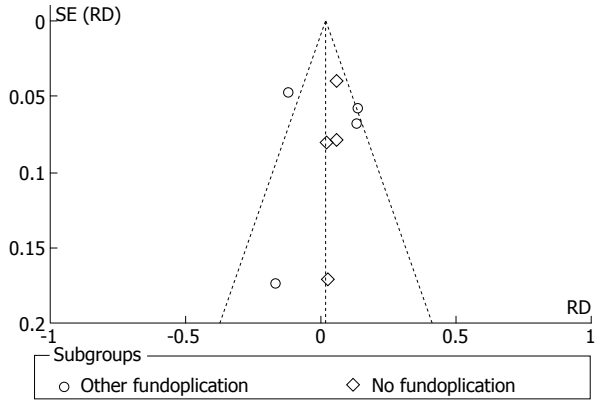


Figure 2 Forest plot. A: Forest plot of the major outcomes in the Dor-other group; B: Forest plot of dysphagia symptoms in both the Dor-other and Dor-no groups; C: Funnel plot of dysphagia symptoms in both the Dor-other and Dor-no groups.

Table 4 Quality assessment of non-random controlled studies in the meta-analysis based on modified Newcastle-Ottawa Scale judgment

Ref.	Selection		Comparability	Outcome assessment		Quality judgment
	1	2	3	4	5	
Dempsey *et al*[12], 2004	*	*	**	*	*	******
Di Martino *et al*[13], 2011	*	*	**	*	*	******
Finley *et al*[14], 2007	*	*	*	*	*	*****
Oelschlager *et al*[15], 2003	*	*	*	*	--	****
Ramacciato *et al*[16], 2005	*	*	*	*	*	*****
Richardson *et al*[20], 2006	*	*	**	*	--	*****
Tapper *et al*[22], 2008	*	--	*	*	*	****
Wright *et al*[24], 2007	*	*	*	*	--	****

Table 5 Pooled outcomes of random controlled studies and non-random controlled studies for postoperative physiological testing and operation-related data

	Studies (*n*)	Participants		Test of heterogeneity		MD (95%CI)	*P* value for effect size
		Dor group	Control group	I^2	*P* value		
LES pressure							
Dor-other group	2	47	43	94%	< 0.0001	-1.02 (-9.90, 7.86)	0.82[1]
Dor-no group	2	58	43	65%	0.09	1.97 (-0.93, 4.86)	0.18[2]
DeMeester score							
Dor-other group	2	40	39	48%	0.17	-7.13 (-18.37, 4.12)	0.21[2]
Dor-no group	1	21	18	Not applicable		-25.00 (-58.40, 8.40)	0.14
Percent total time pH ⩽ 4							
Dor-other group	4	154	142	63%	0.05	0.96 (0.00, 1.91)	0.05[1]
Dor-no group	1	21	18	Not applicable		-7.20 (-13.34, -1.06)	0.02
Surgery time							
Dor-other group	3	138	122	14%	0.31	-5.37 (-7.71, -3.03)	< 0.00001[2]
Dor-no group	2	39	36	0%	0.35	24.14 (7.21, 41.08)	0.005[2]
Hospital stay time							
Dor-other group	4	171	176	94%	< 0.00001	0.10 (-0.59, 0.80)	0.77[1]
Dor-no group	1	22	21	Not applicable		0.00 (-0.15, 0.15)	1.00

[1]Random-effect model; [2]Fixed-effect model. RCT: Random controlled trial.

lasia patients reported no significant difference in the dysphagia frequency score and a significant difference in the dysphagia severity score ($P = 0.465$ and $P = 0.003$, respectively)[24]. No publication bias was observed in the funnel plot of studies when reporting dysphagia in the two comparisons (Figure 2C).

Secondary endpoints: In regards to the other symptoms, one study reported bloating, chest pain, and heartburn recurrence frequency score without any significance difference in the Dor-other group[24]. With regard to quality of life, one multicenter RCT assessed the outcome using an SF-36 questionnaire and ten health-related domains[17]. No significant score difference was observed in the Dor fundoplication group compared with the other fundoplication group in five and seven domains of the total ten domains, respectively. Another prospective study in the Dor-other group reported that the SF-36 score ranged from 0-100, and the two compared types of fundoplication scored 70.5 ± 4.06 and 72.3 ± 4.53 each with a *P* value > 0.5[13].

Postoperative physiologic testing including LES pressure, DeMeester score, and percent total time pH ⩽ 4 displayed no obvious significant difference in the Dor-other group. Considering the relatively high heterogeneity, the random effect model was applied in all three outcomes. In the subgroup analysis of surgery time, Dor fundoplication took significantly less time than the other types of fundoplication, and the estimated hospital stay time was not different in the comparison group. The details are shown in Table 5.

In addition to perforation, other complications were described as follows: Di Martino *et al*[13] reported intra-operatively 1 mucosal tear and 2 cervical subcutaneous emphysema occurrences and postoperatively reported 2 pulmonary atelectasis occurrences in the Dor fundoplication group, intra-operatively 1 pneumothorax occurrence, and postoperatively 1 urinary retention occurrence in the other fundoplication group. Wright *et al*[24] reported 1 urinary retention occurrence in the Dor fundoplication group compared with none in the other fundoplication group. Another 2 studies reported no complications in either group[17,18].

Outcomes in the Dor-no group

Primary endpoints: With respect to clinical regurgitation, two non-RCTs were validly pooled without displaying any significant difference in the Dor-no group (OR = 0.51, 95%CI: 0.09-2.92, $P = 0.32$ and heterogeneity $I^2 = 0\%$)[16,20]. A fixed-effect model was applied in this

analysis. Two studies without an available number of achalasia patients reported no significant differences of dysphagia severity score[14,22].

In the pathological acid reflux analysis, one RCT indicated that Dor fundoplication was associated with an obviously lower pathological acid reflux rate than no fundoplication (OR = 0.11, 95%CI: 0.02-0.59, $P = 0.01$)[19].

Perforation was estimated in 1 RCT and 3 non-RCTs, and a fixed-effect model was used[12,14,16,19]. Subgroups were evaluated with no significant differences in Dor-no group (RD = 0.02, 95%CI: -0.04-0.07, $P = 0.59$ and heterogeneity $I^2 = 0\%$) observed.

Considering dysphagia, no significant symptom relief benefit was found for Dor fundoplication compared with no fundoplication (OR = 1.73, 95%CI: 0.52-5.72, $P = 0.37$ and heterogeneity $I^2 = 0\%$), and a fixed-effects model was used (Figure 2B). Of two studies that did not provide the number of patients, one reported significantly less severe dysphagia in the Dor fundoplication group[22], and the other reported no difference[12].

Secondary endpoints: Concerning other symptoms, there was no obvious difference in heartburn in the Dor-no group in two studies[12,24] and a lower recurrence rate in Dor fundoplication compared with no fundoplication in one study[22]. No significant difference in chest pain was found in 2 studies[12,22], and 1 study reported vomiting without difference and a lower choking rate in the Dor fundoplication group compared with the no fundoplication group[22]. With regard to symptom satisfaction, Dempsey reviewed the consecutive patients with 86% satisfaction in the Dor-no group, which was not significantly different than the Dor group[12]. Tapper and colleagues selectively reviewed their patients and found a slightly higher satisfaction rate in the no fundoplication group compared with the Dor group (89% and 75%, respectively)[22].

Similar to the results in the Dor-other group, postoperative physiologic testing and hospital stay time displayed no obvious significance in the Dor-no group. In addition, Dor fundoplication took significantly more time than no fundoplication on surgery time ($P = 0.005$). Complications were rarely reported in the Dor-no group, except for 2 studies mentioning no complications in this comparison[14,19].

DISCUSSION

Since the description of minimal invasive treatment for achalasia by Shimi *et al*[25], LHM has gained world-wide popularity and is increasingly regarded as the standard treatment for achalasia by surgeons and gastroenterologists. Furthermore, the routine application of fundoplication following LHM has been identified as useful for protection of postoperative GER[4,6,26]. Dor fundoplication, with the advantage of a simple procedure and covering of the mucosa, is being accepted as the first-line type of fundoplication for achalasia in most regions. However, some opponents of Dor fundoplication have reported no significant benefit with regard to the clinical outcomes when Dor fundoplication was added to LHM, and they recommend posterior or even total fundoplication, such as posterior 270° Toupet fundoplication and total 360° Nissen fundoplication, be added to LHM for better long-term outcome[12,27]. Thus, there is no consensus on whether Dor fundoplication is the optimum procedure after LHM for achalasia.

Our group conducted this meta-analysis to provide evidence for fundoplication choice on achalasia surgery. Finally, we found a significantly higher clinical and pathological acid reflux rate in for Dor fundoplication than for other types of fundoplication, although no significant difference was found between Dor fundoplication and no fundoplication. Our results contradict the conventional concept that Dor fundoplication after LHM is the optimal choice for achalasia. However, caution should be taken care to explain the pooled results because of the limitations of our study.

Postoperative GER was the main evaluation used to assess the efficacy of Dor fundoplication for achalasia. GER includes clinical regurgitation symptoms and pathological acid reflux, which was defined as more than 4.2% total time per 24-h period for which pH $\leqslant$ 4 or as a DeMeester score of $\geqslant$ 18 for 24 consecutive hours. Our results demonstrate that Dor fundoplication provides no beneficial clinical regurgitation palliation compared with fundoplication, and, in addition, it leads to a significantly higher clinical regurgitation rate than the other types of fundoplication examined. These results may be explained by the fact that Dor fundoplication may add less resistance than Toupet or Nissen fundoplication, which allows acid to flow through the loose esophagogastric junction more easily. In addition, though the pH testing in two comparison groups indicated no significant difference, the clinical regurgitation symptoms result is supported by the pathological acid reflux outcome, which also demonstrates that Dor fundoplication resulted in more acid reflux than the other fundoplication types. It should be noted that our pooled clinical regurgitation results exclude one study without available data that might affect the outcome[24].

With regard to dysphagia, Dor fundoplication displayed no significant dysphagia relief difference compared with either the other types of fundoplication or no fundoplication. Campos and colleagues suggested that dysphagia relief is independent of whether a fundoplication is performed[2]. Their conclusion is consistent with our pooled outcome that Dor fundoplication did not produce a lower recurrence of dysphagia than the other types of fundoplication, and we also found no obvious difference in postoperative dysphagia when comparing the Dor and Dor-no groups. The relative lack of change in the mucosa fibrosis around the dissected esophagus between the different types of fundoplication may explain this finding, although we have found no significant difference on LES pressure. As the Heller muscle is dissected whether LHM alone or LHM plus fundoplication

is performed, the pressure changes associated with the follow-up procedure may not be significantly different between fundoplication types. Furthermore, a study designed by Rohof on efficacy of treatment for achalasia indicates distensibility of the esophagogastric junction should recommend as better parameter of treatment for achalasia rather than LES pressure[28].

As the most dangerous and latest complication, perforation is the main outcome we focus on. In the pooled outcomes, we failed to find that Dor fundoplication had a lower perforation rate when used for achalasia treatment. As previous studies have reported, perforation was highly related with perioperative therapy, especially pneumatic dilation, and the occurrence is more predicted by the number and duration of dilations[29]. The restricted number of pooled studies and small participant size might decrease the power of these outcomes. In addition, just the fact that observation studies were included may be somewhat responsible for these results.

The surgery time differences can be easily explained by the fact that the more complex surgical procedures and difficulties associated with the other types of complex fundoplication surgeries require more time to perform than Dor fundoplication. The recovery of achalasia patients accounts for many factors: disease itself, surgery, and complications, among others. Our pooled hospital stay time outcome indicates that surgery type has little influence on recovery. Because of the relatively skillful clinicians and the standardized nature of the surgical procedures, no perioperative surgery-related death was found for any surgical type.

Finally, in the sensitivity analysis, the primary pooled estimation of the outcomes is consistent with that of the sensitivity analysis when one study was extracted out, and this result may indicate our pooled results had good quality.

There are some limitations in this meta-analysis: (1) some indirect data acquirement methods were used, such as when dealing with the SD from range; (2) relatively high heterogeneity of data was estimated for the secondary outcomes, especially in postoperative physiological testing. This may be derived from differences in technology used in different regions and countries; (3) RCTs and non-RCTs were pooled for some outcomes because of the lack of available data and studies; and (4) though, we searched for studies without language limitation, the pooled studies were all published in English, which may be responsible for part of the observed heterogeneity.

In summary, we identified a significantly higher recurrence rate of clinical regurgitation and pathological acid reflux for Dor fundoplication than for other types of fundoplication after LHM for achalasia, although no significant difference was found between Dor fundoplication and no fundoplication. Therefore, we conclude that Dor fundoplication after LHM is not the optimum procedure for achalasia and suggest that more attention should be paid on quality of life among different fundoplication approaches.

COMMENTS

Background

Achalasia is generally regarded as an autoimmune esophageal motility disorder resulting from the loss of inhibitory nerve endings in the myenteric plexus of the esophagus, and laparoscopic Heller myotomy (LHM) is commonly used as the main surgical treatment. However, although LHM has been previously shown to have positive long-term outcomes for achalasia patients, gastroesophageal reflux (GER) after LHM is often one of the main failures of treatment.

Research frontiers

In recent years, anterior 180° Dor fundoplication has been recommended after LHM for the prevention of acid reflux. However, LHM alone or LHM plus other types of fundoplication (*e.g.*, posterior 270° Toupet and total 360° Nissen fundoplication) have also been reported to have different benefits compared with LHM plus Dor fundoplication. Thus, there is no consensus on whether Dor fundoplication is the optimum procedure after LHM for the prevention of GER.

Innovations and breakthroughs

Dor fundoplication did not display any obvious benefit in relation to dysphagia and other symptoms versus non-fundoplication or other types of fundoplication surgery. Conversely, the pooled Dor fundoplication results indicated a higher recurrence rate for clinical regurgitation and pathological acid reflux compared with other types of fundoplication (95%CI: 1.25-40.93, and $P = 0.03$ and 95%CI: 1.23-11.72, and $P = 0.02$, respectively), although no significant difference was found between Dor fundoplication and no fundoplication. The results of this meta-analysis indicate that Dor fundoplication after LHM should not be routinely recommended for achalasia.

Applications

This present meta-analysis demonstrates that Dor fundoplication after LHM is not the optimum procedure for achalasia. To prevent postoperative GER, complex types of fundoplication, such as Toupet and Nissen fundoplication, may be added after LHM for the treatment of achalasia.

Peer review

LHM is commonly used to treat achalasia, but GER is a frequent side effect, and an antireflux surgical technique is normally used. The aim of this meta-analysis was to assess Dor-fundoplication compared with non-fundoplication surgical techniques and other types of surgical fundoplication. The paper is well designed and demonstrates the difficulty in assessing and standardizing the published data. The paper is slightly difficult to read, but the graphs and tables facilitate comprehension. This meta-analysis gives the readers relevant reliable data for the selection of fundoplication surgical techniques after LHM.

REFERENCES

1 **Zaninotto G**, Rizzetto C. Endoscopic and surgical management of achalasia. *Eur Surg* 2008; **40**: 146-153 [DOI: 10.1007/s10353-008-0413-1]

2 **Campos GM**, Vittinghoff E, Rabl C, Takata M, Gadenstätter M, Lin F, Ciovica R. Endoscopic and surgical treatments for achalasia: a systematic review and meta-analysis. *Ann Surg* 2009; **249**: 45-57 [PMID: 19106675 DOI: 10.1097/SLA.0b013e31818e43ab]

3 **Lopushinsky SR**, Urbach DR. Pneumatic dilatation and surgical myotomy for achalasia. *JAMA* 2006; **296**: 2227-2233 [PMID: 17090769]

4 **Rosemurgy AS**, Morton CA, Rosas M, Albrink M, Ross SB. A single institution's experience with more than 500 laparoscopic Heller myotomies for achalasia. *J Am Coll Surg* 2010; **210**: 637-645, 645-647 [PMID: 20421021 DOI: 10.1016/j.jamcollsurg.2010.01.035]

5 **Kostic S**, Kjellin A, Ruth M, Lönroth H, Johnsson E, Andersson M, Lundell L. Pneumatic dilatation or laparoscopic cardiomyotomy in the management of newly diagnosed idiopathic achalasia. Results of a randomized controlled trial. *World J Surg* 2007; **31**: 470-478 [PMID: 17308851]

6 **Kashiwagi H**, Omura N. Surgical treatment for achalasia: when should it be performed, and for which patients? *Gen*

Thorac Cardiovasc Surg 2011; **59**: 389-398 [PMID: 21674305 DOI: 10.1007/s11748-010-0765-x]
7 **Mayo D**, Griffiths EA, Khan OA, Szymankiewicz MA, Wakefield CW, Thompson SK. Does the addition of a fundoplication improve outcomes for patients undergoing laparoscopic Heller's cardiomyotomy? *Int J Surg* 2012; **10**: 301-304 [PMID: 22510440 DOI: 10.1016/j.ijsu.2012.04.002]
8 **Hagedorn C**, Jönson C, Lönroth H, Ruth M, Thune A, Lundell L. Efficacy of an anterior as compared with a posterior laparoscopic partial fundoplication: results of a randomized, controlled clinical trial. *Ann Surg* 2003; **238**: 189-196 [PMID: 12894011]
9 **Athanasiou T**, Al-Ruzzeh S, Kumar P, Crossman MC, Amrani M, Pepper JR, Del Stanbridge R, Casula R, Glenville B. Off-pump myocardial revascularization is associated with less incidence of stroke in elderly patients. *Ann Thorac Surg* 2004; **77**: 745-753 [PMID: 14759484]
10 **Wells GA**, Shea B, O'Connell D, Peterson J, Welch V, Losos M, Tugwell P. The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomised studies in meta-analyses. Available from: URL: http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp
11 **Hozo SP**, Djulbegovic B, Hozo I. Estimating the mean and variance from the median, range, and the size of a sample. *BMC Med Res Methodol* 2005; **5**: 13 [PMID: 15840177]
12 **Dempsey DT**, Delano M, Bradley K, Kolff J, Fisher C, Caroline D, Gaughan J, Meilahn JE, Daly JM. Laparoscopic esophagomyotomy for achalasia: does anterior hemifundoplication affect clinical outcome? *Ann Surg* 2004; **239**: 779-785; discussion 785-787 [PMID: 15166957]
13 **Di Martino N**, Brillantino A, Monaco L, Marano L, Schettino M, Porfidia R, Izzo G, Cosenza A. Laparoscopic calibrated total vs partial fundoplication following Heller myotomy for oesophageal achalasia. *World J Gastroenterol* 2011; **17**: 3431-3440 [PMID: 21876635 DOI: 10.3748/wjg.v17.i29.3431]
14 **Finley C**, Clifton J, Yee J, Finley RJ. Anterior fundoplication decreases esophageal clearance in patients undergoing Heller myotomy for achalasia. *Surg Endosc* 2007; **21**: 2178-2182 [PMID: 17514394]
15 **Oelschlager BK**, Chang L, Pellegrini CA. Improved outcome after extended gastric myotomy for achalasia. *Arch Surg* 2003; **138**: 490-495; discussion 495-497 [PMID: 12742951]
16 **Ramacciato G**, D'Angelo FA, Aurello P, Del Gaudio M, Varotti G, Mercantini P, Bellagamba R, Ercolani G. Laparoscopic Heller myotomy with or without partial fundoplication: a matter of debate. *World J Gastroenterol* 2005; **11**: 1558-1561 [PMID: 15770738]
17 **Rawlings A**, Soper NJ, Oelschlager B, Swanstrom L, Matthews BD, Pellegrini C, Pierce RA, Pryor A, Martin V, Frisella MM, Cassera M, Brunt LM. Laparoscopic Dor versus Toupet fundoplication following Heller myotomy for achalasia: results of a multicenter, prospective, randomized-controlled trial. *Surg Endosc* 2012; **26**: 18-26 [PMID: 21789646 DOI: 10.1007/s00464-011-1822-y]
18 **Rebecchi F**, Giaccone C, Farinella E, Campaci R, Morino M. Randomized controlled trial of laparoscopic Heller myotomy plus Dor fundoplication versus Nissen fundoplication for achalasia: long-term results. *Ann Surg* 2008; **248**: 1023-1030 [PMID: 19092347 DOI: 10.1097/SLA.0b013e318190a776]
19 **Richards WO**, Torquati A, Holzman MD, Khaitan L, Byrne D, Lutfi R, Sharp KW. Heller myotomy versus Heller myotomy with Dor fundoplication for achalasia: a prospective randomized double-blind clinical trial. *Ann Surg* 2004; **240**: 405-412; discussion 412-415 [PMID: 15319712]
20 **Richardson WS**, Kennedy CI, Bolton JS. Midterm follow-up evaluation after a novel approach to anterior fundoplication for achalasia. *Surg Endosc* 2006; **20**: 1914-1918 [PMID: 16960666]
21 **Simić AP**, Radovanović NS, Skrobić OM, Raznatović ZJ, Pesko PM. Significance of limited hiatal dissection in surgery for achalasia. *J Gastrointest Surg* 2010; **14**: 587-593 [PMID: 20033338 DOI: 10.1007/s11605-009-1135-9]
22 **Tapper D**, Morton C, Kraemer E, Villadolid D, Ross SB, Cowgill SM, Rosemurgy AS. Does concomitant anterior fundoplication promote dysphagia after laparoscopic Heller myotomy? *Am Surg* 2008; **74**: 626-633; discussion 633-634 [PMID: 18646481]
23 **Torquati A**, Lutfi R, Khaitan L, Sharp KW, Richards WO. Heller myotomy vs Heller myotomy plus Dor fundoplication: cost-utility analysis of a randomized trial. *Surg Endosc* 2006; **20**: 389-393 [PMID: 16437281]
24 **Wright AS**, Williams CW, Pellegrini CA, Oelschlager BK. Long-term outcomes confirm the superior efficacy of extended Heller myotomy with Toupet fundoplication for achalasia. *Surg Endosc* 2007; **21**: 713-718 [PMID: 17332964]
25 **Shimi S**, Nathanson LK, Cuschieri A. Laparoscopic cardiomyotomy for achalasia. *J R Coll Surg Edinb* 1991; **36**: 152-154 [PMID: 1833541]
26 **Patti MG**, Herbella FA. Fundoplication after laparoscopic Heller myotomy for esophageal achalasia: what type? *J Gastrointest Surg* 2010; **14**: 1453-1458 [PMID: 20300876 DOI: 10.1007/s11605-010-1188-9]
27 **Falkenback D**, Johansson J, Oberg S, Kjellin A, Wenner J, Zilling T, Johnsson F, Von Holstein CS, Walther B. Heller's esophagomyotomy with or without a 360 degrees floppy Nissen fundoplication for achalasia. Long-term results from a prospective randomized study. *Dis Esophagus* 2003; **16**: 284-290 [PMID: 14641290]
28 **Rohof WO**, Hirsch DP, Kessing BF, Boeckxstaens GE. Efficacy of treatment for patients with achalasia depends on the distensibility of the esophagogastric junction. *Gastroenterology* 2012; **143**: 328-335 [PMID: 22562023 DOI: 10.1053/j.gastro.2012.04.048]
29 **Smith CD**, Stival A, Howell DL, Swafford V. Endoscopic therapy for achalasia before Heller myotomy results in worse outcomes than heller myotomy alone. *Ann Surg* 2006; **243**: 579-584; discussion 584-586 [PMID: 16632991]

P- Reviewers: Bordas JM, Contini S, Campo SMA, Rajeshwari K
S- Editor: Gou SX **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.v19.i43.7813

World J Gastroenterol 2013 November 21; 19(43): 7813-7815
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Mesenteric vein thrombosis in a patient heterozygous for factor V Leiden and G20210A prothrombin genotypes

Paras Karmacharya, Madan Raj Aryal, Anthony Donato

Paras Karmacharya, Madan Raj Aryal, Anthony Donato, Department of Internal Medicine, Reading Health System, West Reading, PA 19611, United States
Author contributions: Karmacharya P and Aryal MR wrote the initial draft; and Donato A provided the subsequent revisions and final manuscript.
Correspondence to: Paras Karmacharya, MD, Department of Internal Medicine, 6th avenue and Spruce Street, Reading Health System, West Reading, PA 19611, United States. paraskarmacharya@gmail.com
Telephone: 1-484-6288255 Fax: 1-484-6289003
Received: July 26, 2013 Revised: August 27, 2013
Accepted: September 3, 2013
Published online: November 21, 2013

Abstract

Mesenteric venous thrombosis (MVT) is a rare but life threatening form of bowel ischemia. It is implicated in 6%-9% of all cases of acute mesenteric ischemia. The proportion of patients with primary (or idiopathic) MVT varies from 0% to 49%, with a decrease in frequency secondary to more recent availability of newer investigations for hypercoagulability. The presence of factor V Leiden (FVL) and prothrombin G20210A mutations (PGM) have been well documented in these cases. However, there have been scarce case reports describing MVT in heterozygotes of both these mutations occurring simultaneously and its implications on long term management. Our case describes acute MVT in a previously asymptomatic young patient with no prior history of venous thromboembolism. The patient was found to be heterozygous for FVL and PGM and treated with lifelong anticoagulation with warfarin (goal international normalized ratio: 2-3) and avoidance of hormonal contraceptives.

Key words: Mesenteric vein thrombosis; Prothrombin gene; Factor V Leiden; Heterozygous; Anticoagulation; Oral contraceptives

Core tip: The common presence of two thrombophilic defects increases the thrombotic risk several folds above the risk of a single defect and these tend to occur at an earlier age as seen in our case. Also the risk of recurrent thrombosis is significantly increased among these heterozygotes. Indefinite anticoagulation with oral anticoagulants (goal International Normalized Ratio = 2-3) is recommended for high risk patients like our case with thrombosis at unusual sites (*e.g.*, mesenteric vein), and heterozygosity for both factor V Leiden and prothrombin G20210A mutations. These patients should avoid any hormonal therapy and family members should be screened for underlying prothrombotic condition.

Karmacharya P, Aryal MR, Donato A. Mesenteric vein thrombosis in a patient heterozygous for factor V Leiden and G20210A prothrombin genotypes. *World J Gastroenterol* 2013; 19(43): 7813-7815 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7813.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7813

INTRODUCTION

Mesenteric venous thrombosis (MVT) is a rare but life threatening form of bowel ischemia, responsible for 6%-9% of all acute mesenteric ischemia. The presence of factor V Leiden (FVL) and prothrombin G20210A mutations (PGM) have been well documented in these cases. However, there have been scarce case reports describing MVT in heterozygotes of both these mutations occurring simultaneously and its implications on long term management.

CASE REPORT

A 22-year-old Caucasian female presented to the emer-

gency department with progressively worsening, colicky left upper quadrant abdominal pain for 4 d with radiation to her back, associated with some nausea. She denied any history of fever or changes in bowel movements. Past medical history was significant for Polycystic Ovarian syndrome treated with oral contraceptives. She denied personal or family history of thrombosis. Physical exam was significant for tenderness on left upper quadrant of abdomen without guarding, rigidity or rebound tenderness. Complete blood count and electrolytes were within normal limits. A computed tomography (CT) scan of the abdomen revealed possible superior mesenteric vein (SMV) thrombosis without evidence of bowel ischemia (Figure 1). Patient was started on enoxaparin (1 mg/kg twice daily) with bridging to warfarin. Subsequent CT angiography confirmed the initial diagnosis of SMV thrombosis. Surgical intervention was not indicated due to lack of bowel ischemia. Workup for possible vasculitis, including antinuclear antibody, Anti-neutrophil cytoplasmic antibody, titers of hepatitis B, hepatitis C, and Human Immunodeficiency Virus screening were all negative. Hypercoagulable evaluation including protein-C and S, antithrombin Ⅲ, anticardiolipin antibodies were all negative. However she was found to have heterozygous mutations for both prothrombin G20210A and FVL. She was discharged on warfarin with a therapeutic level of anticoagulation. She was advised to have lifelong anticoagulation with warfarin [goal International Normalized Ratio (INR) = 2-3] and avoidance of hormonal contraceptives.

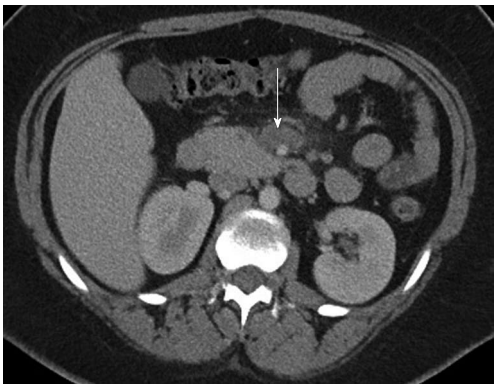


Figure 1 Contrast-enhanced computed tomography scan. It shows decreased attenuation within the superior mesenteric vein (arrow), immediately below the portal confluence, compatible with venous thrombosis.

DISCUSSION

MVT is a rare but potentially life threatening cause of mesenteric ischemia with high recurrence rates[1,2]. It is implicated in 6%-9% of all cases of acute mesenteric ischemia[1,3,4]. Predisposing conditions including myeloproliferative disorders, neoplasia, hereditary hemorrhagic telangiectasia, paroxysmal nocturnal hemoglobinuria, inherited thrombophilias, oral contraceptive pill (OCP) use, pancreatitis, recent abdominal surgery or local intraabdominal infections can be identified in most patients[5]. When no underlying etiology is identified, MVT is described as primary or idiopathic. The proportion of patients with primary (or idiopathic) MVT varies from 0% to 49%, with a decrease in frequency secondary to more recent availability of newer investigations for hypercoagulability. Abdominal pain is the most common symptom, especially with acute thrombosis, whereas chronic MVT usually manifests as portal hypertension or diagnosed incidentally by imaging. The increasing use of CT for the investigation of abdominal pain and anticoagulation for the treatment of acute MVT have improved outcomes in these patients[6]. Surgery and bowel resection may occasionally be needed for patients with bowel infarction, perforation, and peritonitis. The management of patients with chronic MVT is aimed at reducing complications of portal hypertension[4,6].

The present case is of interest in that acute MVT was the initial presentation in a patient with combined heterozygosity for FVL mutation and the G20210A prothrombin gene variation in the face of oral contraceptive use. The association of each of these mutations with thrombotic disease has been well established. Among Caucasian patients presenting with an initial episode of idiopathic deep venous thrombosis, 12%-20% will be found to be heterozygous for the FVL mutation and 6% heterozygous for the prothrombin G20210A gene variation as compared to 6% and 2% respectively, in asymptomatic Caucasian controls[7]. A recent retrospective study by Amitrano *et al*[5] noted a high prevalence of thrombophilic genotypes (75%): FVL (25%), prothrombin *G20210A* gene (25%), and MTHFR prothrombotic defects (50%) in patients with acute mesenteric vein thrombosis. Double heterozygotes of FVL mutation and the prothrombin G20210A gene variation have been shown to be associated with a greater risk of venous thrombosis than either defect alone. Also the age at the first episode of venous thromboembolism in double heterozygotes was significantly younger than those without both gene defects (34.7 years *vs* 40.6 years; $P < 0.01$) in observational and meta-analytic studies. Finally, use of OCP's was associated with a significantly increased risk of thrombosis over those not using them (OR = 16.97, 95%CI: 3.95-72.80)[8-11].

It seems plausible that in our case, MVT was induced by OCPs on the background of her hematological disorders leading to a hypercoagulable state. The common presence of two thrombophilic defects increases the thrombotic risk several folds above the risk of a single defect and these tend to occur at an earlier age which was also seen in our case[10]. Also the risk of recurrent thrombosis is significantly increased among these heterozygotes. Indefinite anticoagulation with oral anticoagulants (with goal INR = 2-3) is recommended for high risk patients like our case with thrombosis at unusual sites (*e.g.*, mesenteric vein), and heterozygosity for both FVL and PGM[9,12,13]. These patients should avoid any hormonal therapy including OCPs due to increased risk of blood clots. It may also be advised to screen the family members for underlying prothrombotic condition, even with a

first episode of idiopathic venous thrombosis[7,11].

REFERENCES

1 **Rhee RY**, Gloviczki P. Mesenteric venous thrombosis. *Surg Clin North Am* 1997; **77**: 327-338 [PMID: 9146716]
2 **Clavien PA**, Dürig M, Harder F. Venous mesenteric infarction: a particular entity. *Br J Surg* 1988; **75**: 252-255 [PMID: 3349333]
3 **Brunaud L**, Antunes L, Collinet-Adler S, Marchal F, Ayav A, Bresler L, Boissel P. Acute mesenteric venous thrombosis: case for nonoperative management. *J Vasc Surg* 2001; **34**: 673-679 [PMID: 11668323 DOI: 10.1067/mva.2001.117331]
4 **Harnik IG**, Brandt LJ. Mesenteric venous thrombosis. *Vasc Med* 2010; **15**: 407-418 [PMID: 20926500 DOI: 10.1177/1358863X10379673]
5 **Amitrano L**, Brancaccio V, Guardascione MA, Margaglione M, Iannaccone L, Dandrea G, Ames PR, Marmo R, Mosca S, Balzano A. High prevalence of thrombophilic genotypes in patients with acute mesenteric vein thrombosis. *Am J Gastroenterol* 2001; **96**: 146-149 [PMID: 11197244 DOI: 10.1111/j.1572-0241.2001.03465.x]
6 **Singal AK**, Kamath PS, Tefferi A. Mesenteric venous thrombosis. *Mayo Clin Proc* 2013; **88**: 285-294 [PMID: 23489453 DOI: 10.1016/j.mayocp.2013.01.012]
7 **Al-Juburi A**, Scott MA, Shah HR, Raufman JP. Heterozygosity for factor V Leiden and G20210A prothrombin genotypes in a patient with mesenteric vein thrombosis. *Dig Dis Sci* 2002; **47**: 601-606 [PMID: 11911349]
8 **Emmerich J**, Rosendaal FR, Cattaneo M, Margaglione M, De Stefano V, Cumming T, Arruda V, Hillarp A, Reny JL. Combined effect of factor V Leiden and prothrombin 20210A on the risk of venous thromboembolism--pooled analysis of 8 case-control studies including 2310 cases and 3204 controls. Study Group for Pooled-Analysis in Venous Thromboembolism. *Thromb Haemost* 2001; **86**: 809-816 [PMID: 11583312]
9 **De Stefano V**, Martinelli I, Mannucci PM, Paciaroni K, Chiusolo P, Casorelli I, Rossi E, Leone G. The risk of recurrent deep venous thrombosis among heterozygous carriers of both factor V Leiden and the G20210A prothrombin mutation. *N Engl J Med* 1999; **341**: 801-806 [PMID: 10477778 DOI: 10.1056/NEJM199909093411104]
10 **Hertzberg MS**, Underwood T, Favaloro EJ. Mesenteric vein thrombosis secondary to combined protein C deficiency and double heterozygosity for factor V Leiden and prothrombin G20210A. *Am J Hematol* 1999; **62**: 199-200 [PMID: 10539890]
11 **Segal JB**, Brotman DJ, Necochea AJ, Emadi A, Samal L, Wilson LM, Crim MT, Bass EB. Predictive value of factor V Leiden and prothrombin G20210A in adults with venous thromboembolism and in family members of those with a mutation: a systematic review. *JAMA* 2009; **301**: 2472-2485 [PMID: 19531787 DOI: 10.1001/jama.2009.853]
12 **Leebeek FW**, Smalberg JH, Janssen HL. Prothrombotic disorders in abdominal vein thrombosis. *Neth J Med* 2012; **70**: 400-405 [PMID: 23123534]
13 **Grouzi E**, Politou M, Douramani P, Merkouri E, Gialeraki A, Brountzos H, Perros G, Travlou A. Portal, splenic and mesenteric vein thrombosis in a patient double heterozygous for factor V Leiden and prothrombin G20210A mutation. *Blood Coagul Fibrinolysis* 2009; **20**: 722-725 [PMID: 19734780 DOI: 10.1097/MBC.0b013e3283306e3c]

P- Reviewers: Goll R, LeitmanM, Matteo F **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.v19.i43.7816

World J Gastroenterol 2013 November 21; 19(43): 7816-7819
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Development of enterohepatic fistula after embolization in ileal gastrointestinal stromal tumor: A case report

Yun Ho Lee, Ja Seol Koo, Chang Ho Jung, Sang Yoon Chung, Jae Joong Lee, Seung Young Kim, Jong Jin Hyun, Sung Woo Jung, Rok Seon Choung, Sang Woo Lee, Jai Hyun Choi

Yun Ho Lee, Ja Seol Koo, Chang Ho Jung, Sang Yoon Chung, Jae Joong Lee, Seung Young Kim, Jong Jin Hyun, Sung Woo Jung, Rok Seon Choung, Sang Woo Lee, Jai Hyun Choi, Division of Gastroenterology, Department of Internal Medicine, Korea University Ansan Hospital, 516 Gojan-dong, Danwon-gu, Ansan-si, Gyeonggi-do 425-707, South Korea

Author contributions: Lee YH, Koo JS, Jung CH, Chung SY, Lee JJ, Kim SY and Hyun JJ reviewed the case and collected the data; Lee YH, Koo JS, Jung SW, Choung RS, Lee SW and Choi JH wrote the paper.

Correspondence to: Ja Seol Koo, MD, PhD, Division of Gastroenterology, Department of Internal Medicine, Korea University Ansan Hospital, 516 Gojan-dong, Danwon-gu, Ansan-si, Gyeonggi-do 425-707, South Korea. jskoo@korea.ac.kr
Telephone: +82-31-4125580 Fax: +82-31-4125582
Received: July 22, 2013 Revised: August 26, 2013
Accepted: September 15, 2013
Published online: November 21, 2013

Abstract

Gastrointestinal stromal tumor (GIST) is a rare mesenchymal tumor of the gastrointestinal tract that has been associated with the formation of fistulas to adjacent organs in few case reports. However, GIST with enterohepatic fistula has not been reported. Here we report the case of an enterohepatic fistula that occurred after embolization of a liver mass originating in the distal ileum. An 87-year-old woman was hospitalized for melena. On initial conventional endoscopy, a bleeding focus in the gastrointestinal tract was not found. Because of massive hematochezia, enteroscopy was performed through the anus. A protruding, ulcerative mass was found in the distal ileum that was suspected to be the source of the bleeding; a biopsy sample was taken. Electrocoagulation was not successful in controlling the bleeding; therefore, embolization was performed. After embolization, the patient developed a high fever and severe abdominal tenderness with rebound tenderness. Follow-up abdominopelvic computed tomography revealed an enterohepatic fistula between the liver and distal ileum. The fistula was treated surgically by segmental resection of the distal ileum and unlooping of the liver mass.

Key words: Gastrointestinal stromal tumor; Enterohepatic fistula; Therapeutic embolization; Bleeding; Ileal gastrointestinal stromal tumor

Core tip: Gastrointestinal stromal tumor (GIST) with fistula is a rare condition, however, it can be seen during treatment. Herein we report a case of an enterohepatic fistula that occurred after therapeutic embolization of liver mass originated from ileal GIST.

Lee YH, Koo JS, Jung CH, Chung SY, Lee JJ, Kim SY, Hyun JJ, Jung SW, Choung RS, Lee SW, Choi JH. Development of enterohepatic fistula after embolization in ileal gastrointestinal stromal tumor: A case report. *World J Gastroenterol* 2013; 19(43): 7816-7819 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7816.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7816

INTRODUCTION

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor originating in the digestive tract. GISTs are thought to arise from the interstitial cells of Cajal in the normal myenteric plexus[1]. GISTs most commonly occur in the stomach (60%), followed by the jejunum and ileum (30%), duodenum (5%), colon and rectum (5%), and, rarely, the esophagus and appendix[2]. However, they account for less than 1% of all gastrointestinal (GI) tumors[3]. It is estimated that up to 6000 new

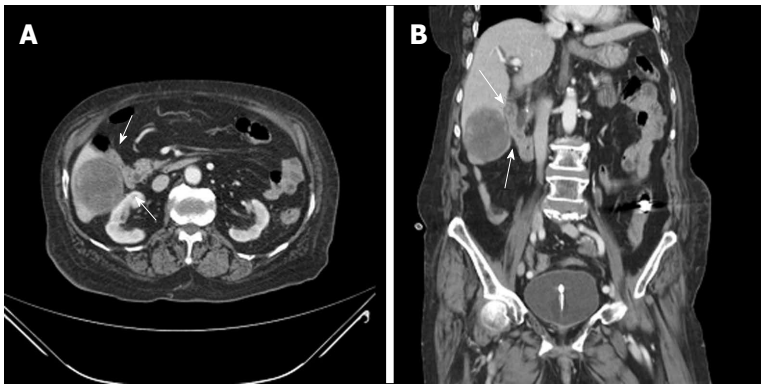


Figure 1 Initial abdominopelvic computed tomography findings. Enhanced computed tomography shows a 5.7 cm × 5.2 cm heterogeneous enhancing liver mass connected with a small mass of the distal ileum (arrows). A: Axial view; B: Coronal view.

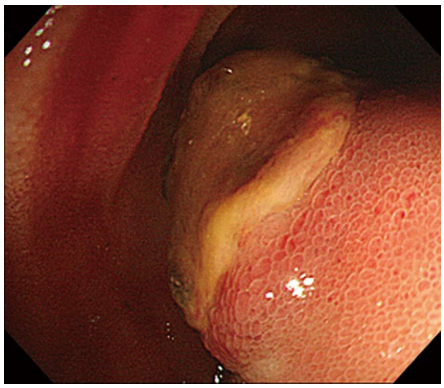


Figure 2 Large protruding mass on the distal ileum (enteroscopic findings). A large protruding mass with ulceration was found on the distal ileum.

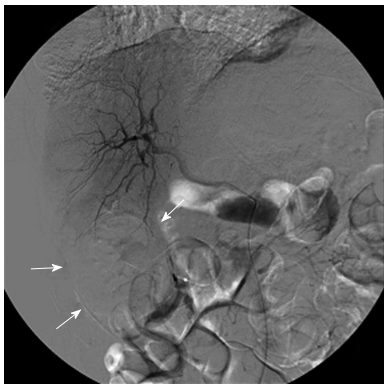


Figure 3 Angiographic findings (post-embolization state). Angiography shows that the hepatic mass (arrows) was not supplied by the right hepatic artery after embolization.

cases are diagnosed in the United States every year[4,5].

Before the late 1990s, these mesenchymal tumors arising in the GI tract were most often classified as smooth muscle tumors or neural tumors. In the 1990s, investigators noted similarities between GIST cells and the interstitial cells of Cajal, a group of cells located in the muscularis propria and around the myenteric plexus throughout the GI tract. These tumors may not cause any symptoms unless they are in a certain location or grow to a certain size. GISTs are often found because they cause bleeding into the GI tract. Other symptoms can result from the mass effect of the tumor causing abdominal discomfort, nausea, vomiting or early satiety. The critical determinants of GIST behavior include tumor size, mitotic rate, and location[6]. The liver is the most common site of metastasis from GISTs, and liver metastases are a major determinant of patient survival[7,8]. Complications such as abscess formation, fistulae or perforation are rare but can be seen during treatment[9,10].

Here we describe the case of a patient with ileal GIST with an enterohepatic fistula caused by tumor necrosis after therapeutic embolization for the control of active bleeding.

CASE REPORT

An 87-year-old woman was admitted to the emergency department because of melena that occurred the day before. She had been taking antihypertensive medication for 7 years and was not taking aspirin or other antiplatelet agents. She had a history of cholecystectomy, total abdominal hysterectomy, and right hemicolectomy due to GI bleeding. At admission, her vital signs were stable; laboratory findings showed anemia with a serum hemoglobin concentration of 7.8 g/dL and leukocytosis with a white blood cell count of 14000/mm^3. A definite bleeding focus was not found on upper endoscopy and total colonoscopy. On a subsequent capsule endoscopy, a small amount of fresh blood was seen in the small bowel but the exact bleeding site was not identified. Abdominopelvic computed tomography (CT) showed a 5.7 cm × 5.2 cm heterogeneous enhancing mass in segments V and VI of the liver (Figure 1) adjacent to the distal ileum and multiple ill-defined low-attenuation lesions that were thought to represent metastases.

On the 9th day of hospitalization, she showed massive hematochezia. Single-balloon enteroscopy was performed through the anus, and a large protruding mass with central ulceration, which was suspected to be the bleeding focus, was found on the distal ileum (Figure 2). After an enteroscopic biopsy of the ileal mass was taken, active bleeding occurred that was not controlled by enteroscopic electrocoagulation. The patient refused surgical treatment for the active ileal bleeding. Therapeutic angiography was performed; however, no extravasation of the contrast was found in the inferior mesenteric or superior mesenteric arteries. Because a liver mass adja-

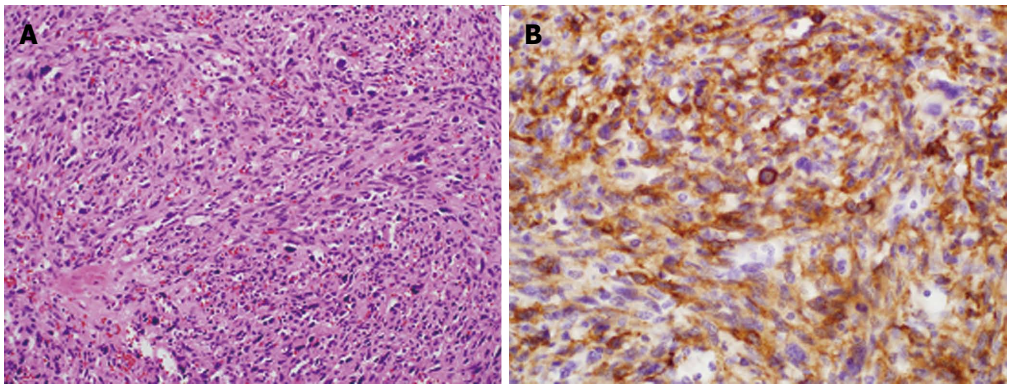


Figure 4 Microscopic findings (Endoscopic biopsy specimen in the ileum). A: Microscopic image of the specimen demonstrating spindle cells (HE, × 200); B: The tumor cells were strongly positive for c-KIT (c-KIT, × 400).

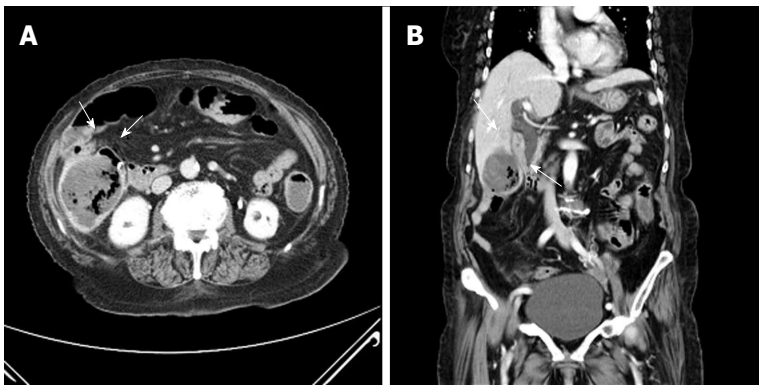


Figure 5 Abdominopelvic computed tomography findings after embolization. Internal necrosis and direct communication (arrows) with the small bowel were identified in the liver mass. A: Axial view; B: Coronal view.

cent to the small bowel had been seen on the abdominopelvic CT, it was suspected that the protruding mass in the distal ileum originated from the suspected malignant liver mass. Based on the assumptions that the bleeding lesion originated from the hepatic mass and was supplied by the hepatic artery, a branch of the right hepatic artery was embolized, and there was no evidence of additional bleeding in the GI tract (Figure 3).

The biopsy specimen showed that the ileal mass was a GIST with more than 20 cells undergoing mitosis per 20 high-power fields (Figure 4). After the embolization, the patient developed fever and right upper quadrant pain. On repeated abdominopelvic CT 5 d after embolization, newly developed internal necrosis and a direct communication with distal ileum were identified in the previously seen liver mass (Figure 5).

Segmental resection of the distal ileum and unlooping of the liver mass were undertaken, and fistula formation between the liver mass and the distal ileum was found intraoperatively. The gross specimen of the resected ileum showed a large fistula orifice that connected with the adjacent liver (Figure 6). Histopathologic examination of the surgical specimen obtained from the resected distal ileum and adjacent liver mass showed that both specimens were identical with GIST on immunohistochemical staining. After the operation, the patient refused chemotherapy and was transferred to a convalescent hospital. She died 6 mo later of multiple organ failure after recurrent GI sepsis.

DISCUSSION

GISTs with intraperitoneal rupture or organ invasion have rarely been reported[11]. Nonetheless, an enterocolic fistula can be caused by prior surgery, malignancy, and infection[12,13]. In similar situations, albeit very rarely, enterohepatic fistulas can develop with GISTs.

We presented a case of an enterohepatic fistula that occurred after embolization of a metastatic liver mass from a malignant GIST. An active bleeding site was not found on initial upper endoscopy and colonoscopy. Abdominopelvic CT demonstrated a 5.7 cm liver mass adjacent to the small bowel.

On initial workup, there was no evidence suggesting that the hepatic mass was the source of the GI bleeding. After enteroscopy by the anal approach because of the patient's massive hematochezia, we found an exophytic mass that was thought to be connected with the hepatic mass and the cause of the bleeding. Therefore, embolization of the right hepatic artery supplying the liver mass was performed. However, within 24 h of arterial embolization, the patient developed fever and right upper quadrant abdominal pain, indicating that fistula formation between the liver mass and distal ileum occurred.

As the patient had undergone several previous abdominal operations, we assumed there was a strong possibility that intraabdominal adhesions were present, specifically between the small bowel and liver. High mitotic rates (> 20 per 20 high power field) also suggested

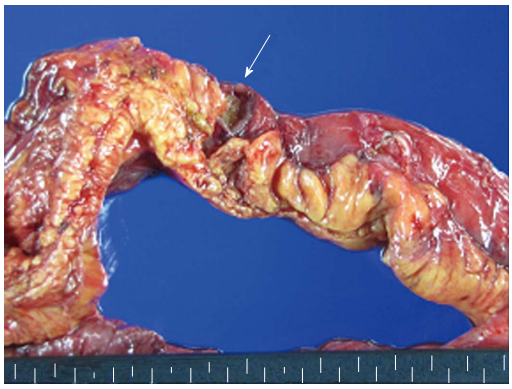


Figure 6 Gross findings of resected segment of the distal ileum. It showed a large fistula orifice (arrow) which connected with the adjacent liver, as proven by food material in the liver mass.

that the tumor was likely to be invasive. Therefore, it was highly probable that the primary ileal tumor directly invaded the adjacent liver and then developed multiple hepatic metastases. Embolization of the right hepatic artery was performed to control the patient's massive hematochezia, and necrosis of the hepatic tumor gradually organized into a fistula.

There have been several cases of GIST with abscess formations, perforations, or various fistulas[9,14-17]. However, GIST with a hepatic fistula is a unique presentation that has not been reported to our knowledge.

Watanabe *et al*[9] presented a similar case of GIST with a vesicocutaneous fistula during treatment with sunitinib. Sunitinib has anti-tumor and anti-angiogenic effects that cause necrosis, similar to our case in which arterial embolization induced visceral necrosis, with a resulting fistula in both cases.

In conclusion, we have reported a case of an enterohepatic fistula that occurred after embolization of a metastatic liver mass in an ileal GIST. It is important that physicians consider the possible complications, such as fistula, perforation and abscess formation, during treatment of highly invasive GISTs.

REFERENCES

1 **Demetri G**, DeMatteo RP. NCCN Task Force report: gastrointestinal stromal tumor (GIST). *J Natl Compr Canc Netw* 2004; **2** Suppl 3: S-25-S-28 [PMID: 23570095]

2 **Miettinen M**, Makhlouf H, Sobin LH, Lasota J. Gastrointestinal stromal tumors of the jejunum and ileum: a clinicopathologic, immunohistochemical, and molecular genetic study of 906 cases before imatinib with long-term follow-up. *Am J Surg Pathol* 2006; **30**: 477-489 [PMID: 16625094 DOI: 10.1097/00000478-200604000-00008]

3 **Stamatakos M**, Douzinas E, Stefanaki C, Safioleas P, Polyzou E, Levidou G, Safioleas M. Gastrointestinal stromal tumor. *World J Surg Oncol* 2009; **7**: 61 [PMID: 19646278 DOI: 10.1186/1477-7819-7-61]

4 **Judson I**, Demetri G. Advances in the treatment of gastrointestinal stromal tumours. *Ann Oncol* 2007; **18** Suppl 10: x20-x24 [PMID: 17761719 DOI: 10.1093/annonc/mdm410]

5 **Corless CL**, Heinrich MC. Molecular pathobiology of gastrointestinal stromal sarcomas. *Annu Rev Pathol* 2008; **3**: 557-586 [PMID: 18039140 DOI: 10.1146/annurev.pathmechdis.3.121806.151538]

6 **Dematteo RP**, Gold JS, Saran L, Gönen M, Liau KH, Maki RG, Singer S, Besmer P, Brennan MF, Antonescu CR. Tumor mitotic rate, size, and location independently predict recurrence after resection of primary gastrointestinal stromal tumor (GIST). *Cancer* 2008; **112**: 608-615 [PMID: 18076015 DOI: 10.1002/cncr.23199]

7 **Bayraktar UD**, Bayraktar S, Rocha-Lima CM. Molecular basis and management of gastrointestinal stromal tumors. *World J Gastroenterol* 2010; **16**: 2726-2734 [PMID: 20533592 DOI: 10.3748/wjg.v16.i22.2726]

8 **Cao G**, Li J, Shen L, Zhu X. Transcatheter arterial chemoembolization for gastrointestinal stromal tumors with liver metastases. *World J Gastroenterol* 2012; **18**: 6134-6140 [PMID: 23155343 DOI: 10.3748/wjg.v18.i43.6134]

9 **Watanabe K**, Otsu S, Morinaga R, Kawano S, Hirashima Y, Sakashita H, Shirao K. Vesicocutaneous fistula formation during treatment with sunitinib malate: Case report. *BMC Gastroenterol* 2010; **10**: 128 [PMID: 21040530 DOI: 10.1186/1471-230X-10-128]

10 **Hur H**, Park AR, Jee SB, Jung SE, Kim W, Jeon HM. Perforation of the colon by invading recurrent gastrointestinal stromal tumors during sunitinib treatment. *World J Gastroenterol* 2008; **14**: 6096-6099 [PMID: 18932293 DOI: 10.3748/wjg.14.6096]

11 **Judson I**. Gastrointestinal stromal tumours (GIST): biology and treatment. *Ann Oncol* 2002; **13** Suppl 4: 287-289 [PMID: 12401703 DOI: 10.1093/annonc/mdf672]

12 **Chun HB**, Baek IH, Lee MS, Kim JB, Shin SR, Kim BC, Jung SY, Kim JW. Jejunocolic fistula associated with an intestinal T cell lymphoma. *Gut Liver* 2011; **5**: 387-390 [PMID: 21927672 DOI: 10.5009/gnl.2011.5.3.387]

13 **Filipovic B**, Randjelovic T, Nikolić G. Gastrojejunocolic fistula as a complication of Billroth II gastrectomy: a case report. *Acta Chir Belg* 2008; **108**: 592-594 [PMID: 19051474]

14 **Kim BH**, Lee JH, Baik du S, Yun SW, Kim JH, Kong JH, Kim SB. [A case of malignant gastrointestinal stromal tumor of ileum with liver abscess]. *Korean J Gastroenterol* 2007; **50**: 393-397 [PMID: 18159178]

15 **Okamoto N**, Yamafuji K, Takeshima K, Hayashi N, Baba H. [A case of gastrointestinal stromal tumor of the duodenum with a huge abscess]. *Nihon Shokakibyo Gakkai Zasshi* 2011; **108**: 1886-1891 [PMID: 22056710]

16 **Shamsaeefar A**, Hosseini SM, Motazedian N, Razmi T. Enterocolic fistula associated with a gastrointestinal stromal tumor. *Indian J Cancer* 2009; **46**: 246-247 [PMID: 19574683 DOI: 10.4103/0019-509X.52965]

17 **Memmi N**, Cipe G, Bektasoglu H, Toydemir T, Kadioglu H, Bozkurt S, Buyukpinarbasili N, Karatepe O, Muslumanoglu M. Perforated gastrointestinal stromal tumor in the jejunum: A rare cause of acute abdomen. *Oncol Lett* 2012; **4**: 1244-1246 [PMID: 23197998]

P- Reviewer: Kozarek R **S- Editor:** Ma YJ **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.v19.i43.7820

World J Gastroenterol 2013 November 21; 19(43): 7820-7824
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

CASE REPORT

Unusual early-stage pancreatic sarcomatoid carcinoma

Chuan-Li Ren, Ping Jin, Chong-Xu Han, Qin Xiao, Dao-Rong Wang, Lin Shi, Da-Xin Wang, Hui Chen

Chuan-Li Ren, Ping Jin, Chong-Xu Han, Laboratory Medicine and Pathology Department, Northern Jiangsu People's Hospital and Clinical Medical College of Yangzhou University, Yangzhou 225001, Jiangsu Province, China
Qin Xiao, Pathology Department, Northern Jiangsu People's Hospital and Clinical Medical College of Yangzhou University, Yangzhou 225001, Jiangsu Province, China
Dao-Rong Wang, Abdominal Surgery Department, Northern Jiangsu People's Hospital and Clinical Medical College of Yangzhou University, Yangzhou 225001, Jiangsu Province, China
Lin Shi, Transonogram Department, Northern Jiangsu People's Hospital and Clinical Medical College of Yangzhou University, Yangzhou 225001, Jiangsu Province, China
Da-Xin Wang, Laboratory Medicine Centre Department, Northern Jiangsu People's Hospital and Clinical Medical College of Yangzhou University, Yangzhou 225001, Jiangsu Province, China
Hui Chen, Geratology Department, Northern Jiangsu People's Hospital and Clinical Medical College of Yangzhou University, Yangzhou 225001, Jiangsu Province, China
Author contributions: Ren CL, Jin P, Xiao Q, Shi L, Chen H and Han CX performed the majority of experiments; Wang DR, Shi L and Wang DX provided vital reagents and analytical tools, and were also involved in editing the manuscript; Ren CL and Chen H designed the study, wrote the manuscript, and provided support for this work.
Supported by China Postdoctoral Science Foundation, No. 2013M541699; Social Development of Jiangsu Province, No. BE2012705 and the Foundation of Northern Jiangsu People's Hospital, No. Yzucms201213
Correspondence to: Hui Chen, MD, Geratology Department, Northern Jiangsu People's Hospital and Clinical Medical College of Yangzhou University, No. 98 Western Nantong Road, Yangzhou 225001, Jiangsu Province, China. chenhuiyangzhou@163.com
Telephone: +86-514-87937516 Fax: +86-514-87937516
Received: August 13, 2013 Revised: September 8, 2013
Accepted: October 13, 2013
Published online: November 21, 2013

Abstract

Sarcomatoid carcinoma of the pancreas (SCP) is a very rare pathological type of carcinoma that usually has a poor prognosis. Its pathogenesis has not been elucidated. We herein report a case of an early-stage SCP involving successful treatment and a good prognosis. The patient was a 48-year-old Chinese man with a 5-mo history of vague abdominal pain. Ultrasonography revealed a 93 mm × 94 mm × 75 mm mass of mixed echogenicity in the tail of the pancreas. Laboratory test results were within the normal range, with the exception of an obviously increased pretreatment neuron-specific enolase level. The plasma transforming growth factor (TGF)β1 and interleukin-11 levels were obviously increased according to enzyme-linked immunosorbent assay. Microscopically, the excised tumor tissue comprised cancer cells and mesenchymal cells. Immunohistochemical analysis was positive for α-1-antichymotrypsin, pan-cytokeratin, cytokeratin 19, cytokeratin 8/18, and vimentin and negative for CD68 and lysozyme. The pathogenetic mechanism of this case shows that TGFβ1 may regulate the epithelial-to-mesenchymal transition in SCP. With early eradication of the tumor and systemic therapy, this patient has been alive for more than 3 years without tumor recurrence or distant metastasis. This case is also the first to show that TGFβ1 may regulate the epithelial-to-mesenchymal transition in early-stage SCP.

Key words: Sarcomatoid carcinoma of the pancreas; Transforming growth factorβ1; Epithelial-to mesenchymal transition; Interleukin-11; Vimentin

Core tip: We herein report a case of an early-stage sarcomatoid carcinoma of the pancreas (SCP) involving successful treatment and a good prognosis. The plasma transforming growth factor (TGF)β1 and interleukin-11 levels were obviously increased according to enzyme-linked immunosorbent assay. Immunohistochemical analysis was positive for pan-cytokeratin, cytokeratin 8/18, and vimentin and negative for CD68 and lysozyme. The pathogenetic mechanism of this case shows that TGFβ1 may regulate the epithelial-to-

mesenchymal transition in SCP. This case is also the first to show that TGFβ1 may regulate the epithelial-to-mesenchymal transition in early-stage SCP.

Ren CL, Jin P, Han CX, Xiao Q, Wang DR, Shi L, Wang DX, Chen H. Unusual early-stage pancreatic sarcomatoid carcinoma. *World J Gastroenterol* 2013; 19(43): 7820-7824 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i43/7820.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i43.7820

INTRODUCTION

Microscopically, sarcomatoid carcinoma of the pancreas (SCP) comprises mostly anaplastic cells and is strikingly sarcoma-like in appearance[1]. SCP may originate from many different organs, such as the pancreas, lung, liver, and esophagus[2-8]. Confirmation of this disease is often based on the pathological diagnosis. Advanced radiographic studies are also good tools with which to support the diagnosis of sarcomatoid carcinoma[2]. Early diagnosis and eradication of the tumor is important for a better prognosis of malignant sarcomatoid carcinomas.

It has been proposed that during malignant progression, carcinoma cells undergo an epithelial-to-mesenchymal transition (EMT), which is a vital step in the formation of pancreatic ductal adenocarcinoma (PDAC)[9]. The etiology of SCP is unknown. The *EML4-ALK* fusion gene was reportedly involved in the development of a sarcomatoid carcinoma of the lung[10]. *ALK* gene amplification is a nonrandom and clonally related event in a subset of pulmonary sarcomatoid carcinomas, but its biologic rationale deserves further investigation[11]. The mechanism of the formation of SCP and its metastasis remains unknown.

CASE REPORT

A 48-year-old Chinese man suffered from vague abdominal pain for 5 mo. He had no evidence of jaundice, hematuria, vomiting, or fever, but abdominal swelling and chest distress were present. He had no smoking or drinking habits, and no history of malignant or other diseases. Ultrasonography revealed a 93 mm × 94 mm × 75 mm mass of mixed echogenicity in the tail of the pancreas (Figure 1). Computed tomography (CT) showed displacement of the retroperitoneal organs by the mass (Figure not shown).

Laboratory test results, including a blood count, serum biochemistry, and urinalysis, were within the normal ranges. The levels of 11 common serum tumor markers, including CA19-9, CEA, and CA242, were normal, except that NSE was obviously increased before any treatment (Table 1). The plasma transforming growth factor (TGF)β1 and Interleukin (IL)-11 levels were higher than those of the healthy controls, patients with PDAC, and patients with pancreatic intraepithelial neoplasias (PanINs) (Table 2).

Table 1 Pretreatment serum tumor markers

Tumor markers	Index	Normal range
CA19-9 (KU/L)	1.21	< 35.00
CA242 (KU/L)	1.14	< 20.00
CA125 (KU/L)	11.71	< 35.00
CA15-3 (KU/L)	2.32	< 35.00
NSE (ng/mL)	23.42	< 13.00
CEA (ng/mL)	0.24	5
Ferritin (ng/mL)	161.47	< 322.00
β-HCG (MIU/mL)	0.12	< 3.00
AFP (ng/mL)	1.07	< 20.00
Free-PSA (ng/mL)	0.32	< 1.00
PSA (ng/mL)	1.53	< 5.00
HGH (ng/mL)	0.36	< 7.50

CA: Cancer antigen; NSE: Neuron-specific enolase; CEA: Carcinoembryonic antigen; β-HCG: β-human chorionic gonadotropin; AFP: α-fetoprotein; PSA: Prostate-specific antigen; HGH: Human growth hormone.

Table 2 Plasma transforming growth factorβ1 and interleukin-11 in sarcomatoid carcinoma before any treatment

	Tumor (pg/mL)	Index (median)	*n*
TGFβ1	SCP	35688	1
	PDAC	10475 (5142-30865)	20
	PanINs	7949 (6655-11404)	10
	HC	6865 (3272-22463)	11
IL-11	SCP	58	1
	HC	22 (10-42)	11

SCP: Sarcomatoid carcinoma of the pancreas; HC: Healthy control; PDAC: Pancreatic ductal adenocarcinoma; PanINs: Pancreatic intraepithelial neoplasias; TGF: Transforming growth factor; IL-11: Interleukin-11.

Surgery was performed, and the tumor was completely resected. The mass measured 10 cm × 8 cm × 3.5 cm and had cystic features after the excision. The section containing the solid tumorous tissue was pale in color. Microscopically, the excised tumor tissue comprised cancer cells and mesenchymal cells, with dispersion of atypical cells and obvious karyokinesis. Some were fusiform in shape and some were multinucleated giant cells (Figure 1A and B). Therefore, SCP was pathologically diagnosed. The neighboring lymph nodes and incisal margin were free of tumor cells. Immunohistochemical study results showed that the tumor cells were positive for vimentin, α-1-antichymotrypsin (AACT), cytokeratin 19, cytokeratin 18 (Figure 1C), and pan-cytokeratin (Figure 1D) and negative for CD68 and lysozyme (data not shown). Thus, an early-stage SCP was diagnosed.

The preoperative diagnosis was cystadenoma in the tail of the pancreas. Seven months after surgical excision, there was no evidence of tumor recurrence or metastasis. Digital subtraction angiography interventional chemotherapy was then implemented. Gemcitabine (1.4 g), oxaliplatin (150 mg), and floxuridine (1.0 g) were intravenously injected *via* the superior mesenteric artery and celiac trunk artery. After 28 mo of follow-up, a

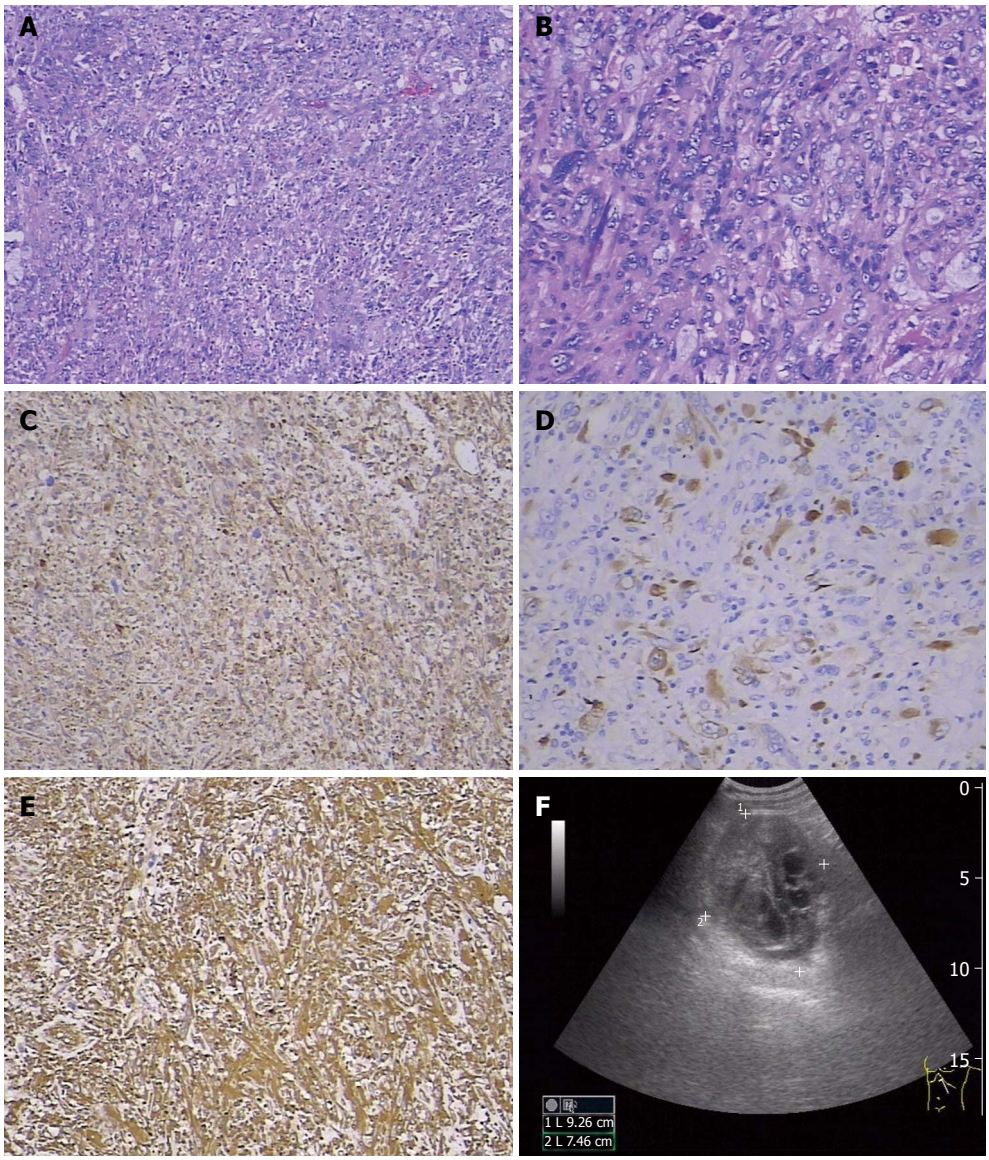


Figure 1 Hematoxylin and eosin stained sections, immunohistochemical test and ultrasonography diagnosis. A: Histologic findings of the tumor; the morphology of sarcomatoid carcinoma of the pancreas (SCP) is shown (hematoxylin and eosin, × 100); B: Microscopically, the excised tumor tissue comprised cancer cells and mesenchymal cells, with dispersion of atypical cells and obvious karyokinesis (hematoxylin and eosin, × 200); C: Widely diffuse immunohistochemical staining for the epithelial marker cytokeratin 18 (× 100); D: Heterogeneous immunohistochemical staining for the epithelial marker pan-cytokeratin (Pan-CK) (× 100); E: Widely diffuse immunohistochemical staining for the mesenchymal marker vimentin (× 100); F: Ultrasonography revealed a 93 mm × 94 mm × 75 mm mass of mixed echogenicity in the tail of the pancreas.

routine check-up and CT scan revealed that the patient was in good condition and free of tumor recurrence and metastasis. Because the patient had the opportunity to be treated in the early stage of the disease, he is in good condition and has been alive for more than 3 years without tumor recurrence or metastasis.

DISCUSSION

Sarcomatoid carcinoma is a rare and very aggressive malignant tumor comprising a mixture of carcinomatous and sarcomatous elements[12]. Areas of spindle cells arranged in a storiform pattern were present[13]. The tumor demonstrated cellular patterns similar to those present in tumors of mesenchymal origin in the case. In the present case, many cells undergoing heterotypic division were seen in the tissue specimen, and karyokinesis was frequent. Some cells were fusiform in shape, and some were pleomorphic giant cells. This change into pleomorphic giant cells was the most frequent sarcomatoid transformation encountered[1]. Compared with ordinary pancreatic carcinomas, malignant giant cell tumors of the pancreas appear to have a distinctive behavior characterized by local invasiveness, a reluctance to metastasize, and a more favorable prognosis when resected[1]. Immunohistochemical study results showed that the tumor cells were positive for vimentin, AACT, pan-cytokeratin, cytokeratin 19, and cytokeratin 8/18 and negative for CD68 and lysozyme. Some authors reported that the tumor cells in sarcomatoid carcinoma were positive for

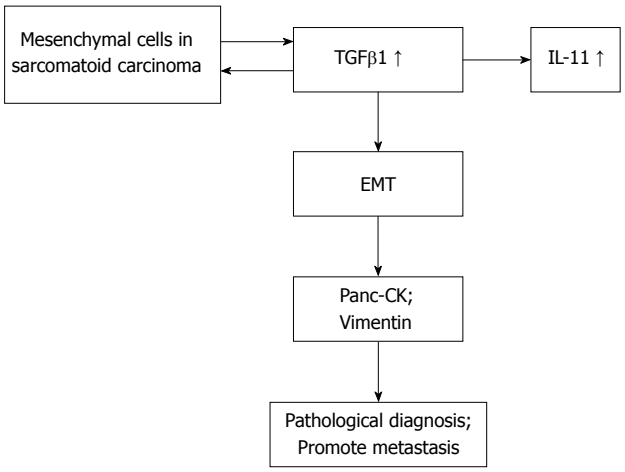


Figure 2 Mechanism of transforming growth factorβ1 regulating the epithelial-to-mesenchymal transition of sarcomatoid carcinoma of the pancreas. TGF: Transforming growth factor; EMT: Epithelial-to-mesenchymal transition; IL-11: Interleukin-11.

CK, S-100 protein, α1-antitrypsin, α1-chymotrypsin, anti-CA19-9, and SMA. No cells were positive for vimentin or desmin[14]. Vimentin-positive tumor cells are of mesenchymal origin in sarcomatoid carcinoma and are also seen in inflammatory myofibroblastic tumor of the prostate, another rare malignant disease[15]. In the present case, both epithelial and mesenchymal markers were positive. The process of EMT may play an important role in the formation of SCP. TGFβ signaling plays a dual role in oncogenesis. TGFβ can sometimes function as a tumor suppressor gene that inhibits the proliferation of normal epithelial cells, while in other tumor types it functions as an oncogenic gene. This dual function implies that the activity of TGFβ is highly dependent on the cellular context, pathological type, and specific environment[16-21]. In this case, the TGFβ1 level was markedly higher than those in patients with PDAC and PanINs and in HCs. TGFβ1 may regulate the EMT pathway in pancreas cells and promote the formation of SCP. The plasma IL-11 level in the present patient was obviously higher than that of the healthy controls. IL-11 is a TGFβ target gene. IL-11 stimulates the production of the osteoclastogenic factors RANKL and granulocyte macrophage-colony stimulating factor in osteoblasts. Induction of IL-11 and CTGF expression by TGFβ is mediated by the SMAD pathway[22]. TGFβ1 could be an important driving force during the sarcomatoid transdifferentiation of clear cell renal cell carcinoma[23]. The combination of early diagnosis of sarcomatoid carcinoma, eradication of the tumor, and systemic therapy may provide a chance of a good prognosis. Whether postoperative patients in the early tumor stage require chemotherapy may be controversial. High levels of NSE, TGFβ1, and IL-11 in the serum or plasma may help in the early diagnosis of SCP. TGFβ1 may play an important role in tumor metastasis (Figure 2) and some papers support our hypothesis[24,25]. In view of the complex biologic behavior of SCP, continued real-time monitoring of the clinical course of the disease is strongly recommended.

REFERENCES

1 **Alguacil-Garcia A**, Weiland LH. The histologic spectrum, prognosis, and histogenesis of the sarcomatoid carcinoma of the pancreas. *Cancer* 1977; **39**: 1181-1189 [PMID: 912652]
2 **Mi B**, Wan W, Yu C, You X, Xu Q. Sarcomatoid hepatocellular carcinoma diagnosed by FDG PET/CT. *Clin Nucl Med* 2011; **36**: 925-927 [PMID: 21892049 DOI: 10.1097/RLU.0b013e31821c9939]
3 **Raza MA**, Mazzara PF. Sarcomatoid carcinoma of esophagus. *Arch Pathol Lab Med* 2011; **135**: 945-948 [PMID: 21732788]
4 **Jung LY**, Jeon SY, Kim SR, Chung MJ, Lee YC. Pulmonary sarcomatoid carcinoma accompanying duodenal involvement. *Am J Respir Crit Care Med* 2012; **185**: 899-900 [PMID: 22505760 DOI: 10.1164/ajrccm.185.8.899]
5 **Lu HS**, Gan MF, Zhou T, Wang SZ. Sarcomatoid thymic carcinoma arising in metaplastic thymoma: a case report. *Int J Surg Pathol* 2011; **19**: 677-680 [PMID: 20034984 DOI: 10.1177/1066896909355458]
6 **Shuch B**, Bratslavsky G, Linehan WM, Srinivasan R. Sarcomatoid renal cell carcinoma: a comprehensive review of the biology and current treatment strategies. *Oncologist* 2012; **17**: 46-54 [PMID: 22234634 DOI: 10.1634/theoncologist.2011-0227]
7 **Molina AM**, Tickoo SK, Ishill N, Trinos MJ, Schwartz LH, Patil S, Feldman DR, Reuter VE, Russo P, Motzer RJ. Sarcomatoid-variant renal cell carcinoma: treatment outcome and survival in advanced disease. *Am J Clin Oncol* 2011; **34**: 454-459 [PMID: 21127411 DOI: 10.1097/COC.0b013e3181f47aa4]
8 **Shuch B**, Said J, LaRochelle JC, Zhou Y, Li G, Klatte T, Pouliot F, Kabbinavar FF, Belldegrun AS, Pantuck AJ. Histologic evaluation of metastases in renal cell carcinoma with sarcomatoid transformation and its implications for systemic therapy. *Cancer* 2010; **116**: 616-624 [PMID: 19998348 DOI: 10.1002/cncr.24768]
9 **Rhim AD**, Mirek ET, Aiello NM, Maitra A, Bailey JM, McAllister F, Reichert M, Beatty GL, Rustgi AK, Vonderheide RH, Leach SD, Stanger BZ. EMT and dissemination precede pancreatic tumor formation. *Cell* 2012; **148**: 349-361 [PMID: 22265420 DOI: 10.1016/j.cell.2011.11.025]
10 **Alì G**, Proietti A, Niccoli C, Pelliccioni S, Borrelli N, Giannini R, Lupi C, Valetto A, Bertini V, Lucchi M, Mussi A, Fontanini G. EML4-ALK translocation in both metachronous second primary lung sarcomatoid carcinoma and lung adenocarcinoma: a case report. *Lung Cancer* 2013; **81**: 297-301 [PMID: 23664446 DOI: 10.1016/j.lungcan.2013.03.016]
11 **Pelosi G**, Gasparini P, Cavazza A, Rossi G, Graziano P, Barbareschi M, Perrone F, Barberis M, Takagi M, Kunimura T, Yamada T, Nakatani Y, Pastorino U, Scanagatta P, Sozzi G, Garassino M, De Braud F, Papotti M. Multiparametric molecular characterization of pulmonary sarcomatoid carcinoma reveals a nonrandom amplification of anaplastic lymphoma kinase (ALK) gene. *Lung Cancer* 2012; **77**: 507-514 [PMID: 22705117 DOI: 10.1016/j.lungcan.2012.05.093]
12 **De la Riva S**, Muñoz-Navas MA, Betés M, Súbtil JC, Carretero C, Sola JJ. Sarcomatoid carcinoma of the pancreas and congenital choledochal cyst. *Gastrointest Endosc* 2006; **64**: 1005-1006; discussion 1006 [PMID: 17140915 DOI: 10.1016/j.gie.2006.06.004]
13 **Cresson DH**, Reddick RL. Sarcomatoid carcinoma of the pancreas presenting as gastric carcinoma: clinicopathologic and ultrastructural findings. *J Surg Oncol* 1987; **36**: 268-274 [PMID: 3695533 DOI: 10.1002/jso.2930360411]
14 **Higashi M**, Takao S, Sato E. Sarcomatoid carcinoma of the pancreas: a case report with immunohistochemical study. *Pathol Int* 1999; **49**: 453-456 [PMID: 10417690 DOI: 10.1046/j.1440-1827.1999.00877.x]

15 **Liu C**, Zhao X, Zhao Z, Lu P, Jin F, Li G. Malignant inflammatory myofibroblastic tumor of the prostate. *J Clin Oncol* 2013; **31**: e144-e147 [PMID: 23401456 DOI: 10.1200/JCO.2012.44.4851]
16 **Massagué J**. TGFβ signalling in context. *Nat Rev Mol Cell Biol* 2012; **13**: 616-630 [PMID: 22992590 DOI: 10.1038/nrm3434]
17 **Akhurst RJ**. Large- and small-molecule inhibitors of transforming growth factor-beta signaling. *Curr Opin Investig Drugs* 2006; **7**: 513-521 [PMID: 16784021]
18 **Cha Y**, Kim DK, Hyun J, Kim SJ, Park KS. TCEA3 binds to TGF-beta receptor I and induces Smad-independent, JNK-dependent apoptosis in ovarian cancer cells. *Cell Signal* 2013; **25**: 1245-1251 [PMID: 23357533 DOI: 10.1016/j.cellsig.2013.01.016]
19 **Arteaga CL**. Inhibition of TGFbeta signaling in cancer therapy. *Curr Opin Genet Dev* 2006; **16**: 30-37 [PMID: 16377175 DOI: 10.1016/j.gde.2005.12.009]
20 **Massagué J**. TGFbeta in Cancer. *Cell* 2008; **134**: 215-230 [PMID: 18662538 DOI: 10.1016/j.cell.2008.07.001]
21 **Seoane J**. The TGFBeta pathway as a therapeutic target in cancer. *Clin Transl Oncol* 2008; **10**: 14-19 [PMID: 18208788 DOI: 10.1007/s12094-008-0148-2]
22 **Kang Y**, He W, Tulley S, Gupta GP, Serganova I, Chen CR, Manova-Todorova K, Blasberg R, Gerald WL, Massagué J. Breast cancer bone metastasis mediated by the Smad tumor suppressor pathway. *Proc Natl Acad Sci USA* 2005; **102**: 13909-13914 [PMID: 16172383 DOI: 10.1073/pnas.0506517102]
23 **Boström AK**, Möller C, Nilsson E, Elfving P, Axelson H, Johansson ME. Sarcomatoid conversion of clear cell renal cell carcinoma in relation to epithelial-to-mesenchymal transition. *Hum Pathol* 2012; **43**: 708-719 [PMID: 21992819 DOI: 10.1016/j.humpath.2011.06.019]
24 **Bonnomet A**, Syne L, Brysse A, Feyereisen E, Thompson EW, Noël A, Foidart JM, Birembaut P, Polette M, Gilles C. A dynamic in vivo model of epithelial-to-mesenchymal transitions in circulating tumor cells and metastases of breast cancer. *Oncogene* 2012; **31**: 3741-3753 [PMID: 22120722 DOI: 10.1038/onc.2011.540]
25 **Calon A**, Espinet E, Palomo-Ponce S, Tauriello DV, Iglesias M, Céspedes MV, Sevillano M, Nadal C, Jung P, Zhang XH, Byrom D, Riera A, Rossell D, Mangues R, Massagué J, Sancho E, Batlle E. Dependency of colorectal cancer on a TGF-β-driven program in stromal cells for metastasis initiation. *Cancer Cell* 2012; **22**: 571-584 [PMID: 23153532 DOI: 10.1016/j.ccr.2012.08.013]

P- Reviewers: Azuma YT, Maeda S, Osawa S
S- Editor: Qi Y **L- Editor:** Rutherford A **E- Editor:** Liu XM

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
www.wjgnet.com

World J Gastroenterol 2013 November 21; 19(43): I-VI
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

INSTRUCTIONS TO AUTHORS

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 1352 experts in gastroenterology and hepatology from 64 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 42 OA clinical medical journals, including 41 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: wjg@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 wjg@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, Viscer-

al, 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 boldfaced 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 boldfaced 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 Pixudiarrhoea. *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.00000 35706.28494.09]

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
4 3>
9 771007 932045